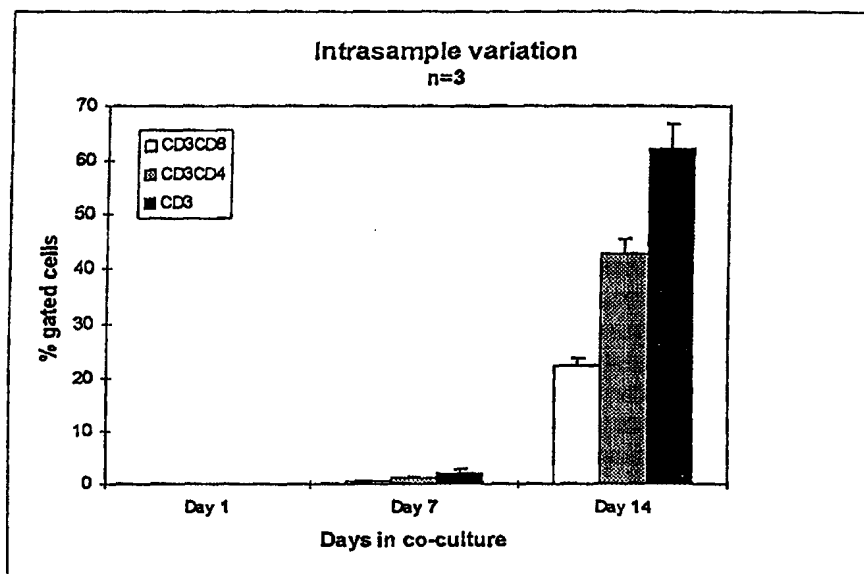




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 5/00		A2	(11) International Publication Number: WO 00/27999
			(43) International Publication Date: 18 May 2000 (18.05.00)
(21) International Application Number: PCT/US99/26795 (22) International Filing Date: 12 November 1999 (12.11.99) (30) Priority Data: 60/107,972 12 November 1998 (12.11.98) US (71) Applicants (for all designated States except US): CYTOMATRIX, LLC [US/US]; 50 Cummings Park, Woburn, MA 01801 (US). THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): ROSENZWEIG, Michael [US/US]; Cytomatrix, LLC, 50 Cummings Park, Woburn, MA 01801 (US). PYKETT, Mark, J. [US/US]; Cytomatrix, LLC, 50 Cummings Park, Woburn, MA 01801 (US). SCADDEN, David, T. [US/US]; Massachusetts General Hospital East, 13th Street, Building 149, Charlestown, MA 02129 (US). POZNANSKY, Mark, C. [US/US]; Massachusetts General Hospital East, 13th Street, Building 149, Charlestown, MA 02129 (US). (74) Agent: GATES, Edward, R.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).		(81) Designated States: AU, CA, CN, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published Without international search report and to be republished upon receipt of that report.	

(54) Title: LYMPHOID TISSUE-SPECIFIC CELL PRODUCTION FROM HEMATOPOIETIC PROGENITOR CELLS IN THREE-DIMENSIONAL DEVICES



(57) Abstract

The invention relates to a method for lymphoid tissue-specific cell production from hematopoietic progenitor cells in unique, three-dimensional culture devices, in the presence of lymphoreticular stromal cells and in the absence of exogenously added growth factors. The resulting differentiated progeny. The lymphoid tissue-specific cells may be isolated at any sequential stage of differentiation and further expanded. The lymphoid tissue-specific cells also may be genetically altered at any stage of the process.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**LYMPHOID TISSUE-SPECIFIC CELL PRODUCTION FROM
HEMATOPOIETIC PROGENITOR CELLS IN THREE-DIMENSIONAL DEVICES**

Related Applications

This application claims priority under 35 U.S.C. §119 from Provisional U.S. Patent Application Serial No. 60/107,972 filed on November 12, 1998, entitled LYMPHOID TISSUE-SPECIFIC CELL PRODUCTION FROM HEMATOPOIETIC PROGENITOR CELLS IN THREE-DIMENSIONAL DEVICES. The contents of the provisional application are hereby expressly incorporated by reference.

Field of the Invention

The invention pertains to the co-culture of hematopoietic progenitor cells and lymphoreticular stromal cells in three-dimensional devices, resulting in unexpectedly high numbers of lymphoid tissue-specific cell progeny.

Background of the Invention

A characteristic of the immune system is the specific recognition of antigens. This includes the ability to discriminate between self and non-self antigens and a memory-like potential that enables a fast and specific reaction to previously encountered antigens. The vertebrate immune system reacts to foreign antigens with a cascade of molecular and cellular events that ultimately results in the humoral and cell-mediated immune response.

The major pathway of the immune defense involving antigen-specific recognition commences with the trapping of the antigen by antigen presenting cells (APCs), such as dendritic cells or macrophages, and the subsequent migration of these cells to lymphoid organs (e.g., thymus). There, the APCs present antigen to subclasses of T cells classified as mature T helper cells. Upon specific recognition of the presented antigen, the mature T helper cells can be triggered to become activated T helper cells. The activated T helper cells regulate both the humoral immune response by inducing the differentiation of mature B cells to antibody producing plasma cells and the cell-mediated immune response by activation of mature cytotoxic T cells.

The thymus has been shown to be an obligatory factor in T cell differentiation of hematopoietic cells. Based upon the murine model, it is believed that the presence of a three dimensional organ is required, as *in vitro* models that do not include the thymus and a three dimensional structure fail to support T cell lymphopoiesis (Owen JJ, et al., *Br Med Bull.*,

1989, 45:350-360). The process of differentiation, however, appears to begin prior to progenitor cells contacting the thymus.

Primitive hematopoietic progenitors in the fetal liver or bone marrow give rise to lineage committed cells, including progenitors committed to the T lymphoid lineage. These most immature cells are identified by the surface expression of CD34. T cell lineage committed cells express CD34, but no discrete expression of other epitopes found only on T cell progenitors has been described. Further, T lymphocyte differentiation normally occurs via a series of discrete developmental stages. Primitive progenitor cells which do not express lymphocyte specific cell surface markers (CD34+ CD3- CD4- CD8-) migrate to the thymus where they acquire, through a series of maturational events, the phenotype CD34- CD3- CD4+ CD8-. These cells then mature into double positive CD4+ CD8+ cells, most of which are CD3+, although CD3 expression is not universally detectable. These cells further undergo both positive and negative selection, and mature to develop into single positive T cells (CD4+ CD8- or CD4- CD8+). These cells ultimately migrate into the peripheral circulation as naive T cells.

T cell disorders and diseases represent major health problems. Recent progress has been made using gene therapy to treat conditions involving T lymphocytes, including AIDS. This has fostered increased interest in the development of laboratory techniques that allow *in vitro* evaluations of potential genetic therapies for these conditions.

The understanding of T cell differentiation has been hampered by the limited availability of technologies which permit *in vitro* T cell differentiation. To date, T cell differentiation studies have been largely confined to the SCID-hu mouse *in vivo* model. *In vitro* technologies have been based on thymic explant studies and primate thymic monolayers. In a recent advance, primate thymic stroma cultures have been shown to provide an expedient, although inefficient, system for examining T cell development, enabling *in vitro* T cell differentiation in a reproducible manner. However, the purity and number of T cells generated this way, as well as the relatively short half-life of the cultures, generally results in limited applicability to more advanced studies of T cell differentiation and function.

Summary of the Invention

The invention, in one important part, involves improved methods for culturing hematopoietic progenitor cells that direct their development toward lymphoid tissue-specific lineages without the addition of exogenous growth factors. Thus, one aspect of the invention is the culture of hematopoietic progenitor cells to generate progeny committed to a specific

lineage. Another aspect is an improvement in the rate and the number of differentiated progeny that can be obtained from a sample of hematopoietic progenitor cells.

We describe herein a system that takes advantage of biocompatible, open-pore, three-dimensional matrices, and uses human and non-human lymphoreticular stromal cells to provide the appropriate conditions for the expansion and differentiation of human and non-human hematopoietic progenitor cells toward a specific cell lineage. T lymphocytes, for example, derived from these cultures respond normally to a variety of stimuli and express the diversity of markers expected of mature T cells.

This system provides significant advantages over existing techniques. For example, it can provide for the rapid generation of a large number of differentiated progeny necessary for laboratory analysis and/or therapeutic uses, including for *in vitro* testing of potential gene therapy strategies or for reinfusion into subjects *in vivo*. The matrix itself can be implanted into subjects for *in vivo* studies of hematopoietic cell growth. The system also can reasonably replicate the complex process of hematopoietic cell maintenance, expansion and/or differentiation toward a specific lineage.

Surprisingly, according to the invention, it has been discovered that hematopoietic progenitor cells co-cultured with lymphoreticular stromal cells in a porous solid scaffold, without the addition of exogenous growth agents, generate at a fast rate an unexpectedly high number of functional, differentiated progeny of a lymphoid-specific lineage. The lymphoid tissue from which lymphoreticular stromal cells are derived helps determine the lineage-commitment hematopoietic progenitor cells undertake, resulting in the lineage-specificity of the differentiated progeny. Also surprising, according to the invention, is the discovery that lesser amounts of nonlymphoid cells (i.e. myelo-monocytic cells) are generated from the co-culture of hematopoietic progenitor cells and lymphoreticular stromal cells in a porous solid scaffold of the invention when compared to existing methodology. Thus, the present invention permits for the rapid generation of a large number of differentiated, lymphoid-specific cells from a relatively small number of hematopoietic progenitor cells. Such results were never before realized using known art methodologies (e.g., as in U.S. Patent No. 5,677,139 by Johnson *et al.*, which describes the *in vitro* differentiation of CD3⁺ cells on primate thymic stroma monolayers, or as in U.S. Patent No. 5,541,107 by Naughton *et al.*, which describes a three-dimensional bone marrow cell and tissue culture system).

According to one aspect of the invention, a method for *in vitro* production of lymphoid tissue-specific cells is provided. The method involves introducing an amount of

hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix. The hematopoietic progenitor cells and the lymphoreticular stromal cells are then co-cultured. The amount of the lymphoreticular stromal cells utilized is sufficient to support the growth and differentiation of the hematopoietic progenitor cells. In one embodiment, co-culturing occurs under conditions sufficient to produce at least a 10-fold increase in the number of lymphoid tissue origin cells. In preferred embodiments, co-culturing occurs under conditions sufficient to produce at least a 20, 50, 100, 200, 300 or 400 -fold increase in the number of lymphoid tissue origin cells. In some embodiments, after the co-culturing, harvesting of the lymphoid tissue origin cells may be performed.

In certain embodiments, the hematopoietic progenitor cells may be pluripotent stem cells, multipotent progenitor cells and/or progenitor cells committed to specific hematopoietic lineages. The progenitor cells committed to specific hematopoietic lineages may be of T cell lineage, B cell lineage, dendritic cell lineage, Langerhans cell lineage and/or lymphoid tissue-specific macrophage cell lineage.

The hematopoietic progenitor cells may be derived from a tissue such as bone marrow, peripheral blood (including mobilized peripheral blood), umbilical cord blood, placental blood, fetal liver, embryonic cells (including embryonic stem cells), aortal-gonadal-mesonephros derived cells, and lymphoid soft tissue. Lymphoid soft tissue includes the thymus, spleen, liver, lymph node, skin, tonsil and Peyer's patches. In other embodiments, the lymphoreticular stromal cells may be also derived from at least one of the foregoing lymphoid soft tissues. In preferred embodiments, the lymphoreticular stromal cells are thymic stromal cells and the multipotent progenitor cells and/or committed progenitor cells are committed to a T cell lineage. In other embodiments, the hematopoietic progenitor cells and/or the lymphoreticular stromal cells may be genetically altered.

In one important embodiment of the invention, the hematopoietic progenitor cells are of human origin and the lymphoreticular stromal cells are also of human origin. In another embodiment, the hematopoietic progenitor cells are of human origin and the lymphoreticular stromal cells are of non-human origin. In preferred embodiments, non-human lymphoreticular stromal cells are of murine origin.

In certain embodiments, the lymphoreticular stromal cells are seeded to the matrix at the same time as the hematopoietic progenitor cells. In other embodiments, the

lymphoreticular stromal cells are seeded to the matrix prior to inoculating the hematopoietic progenitor cells.

The porous matrix can be one that is an open cell porous matrix having a percent open space of at least 50%, and preferably at least 75%. In one embodiment the porous solid matrix has pores defined by interconnecting ligaments having a diameter at midpoint, on average, of less than 150 μm . Preferably the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material, the metal coating being selected from the group consisting of tantalum, titanium, platinum (including other metals of the platinum group), niobium, hafnium, tungsten, and combinations thereof. In preferred embodiments, whether the porous solid matrix is metal-coated or not, the matrix is coated with a biological agent selected from the group consisting of collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitrogen, antibodies and fragments thereof, functional equivalents of these factors (including fragments thereof), and combinations thereof. Most preferably the metal coating is tantalum coated with a biological agent. In certain other embodiments, the porous solid matrix having seeded hematopoietic progenitor cells and their progeny, and lymphoreticular stromal cells, is impregnated with a gelatinous agent that occupies pores of the matrix.

The preferred embodiments of the invention are solid, unitary macrostructures, i.e. not beads or packed beads. They also involve nonbiodegradable materials.

According to any of the foregoing embodiments, the method of the invention can include culturing the cells in an environment that is free of hematopoietic progenitor cell survival and proliferation factors such as interleukins 3, 6 and 11, stem cell ligand and FLT-3 ligand. Still another embodiment of the invention is performing the co-culturing of the hematopoietic progenitor cells and the lymphoreticular stromal cells in an environment that is free altogether of stromal cell conditioned medium and exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion and/or differentiation, other than serum.

As will be understood, according to the invention, it is possible now to co-culture hematopoietic progenitor cells and lymphoreticular stromal cells in an environment that is free of exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion and/or differentiation for as little as 7 days and to obtain large numbers of differentiated progeny of a specific lineage.

According to any of the foregoing embodiments, the method of the invention can include co-culturing of the hematopoietic progenitor cells and the lymphoreticular stromal cells with an exogenously added agent selected from the group consisting of stromal cell conditioned medium, and a hematopoietic growth factor that promotes hematopoietic cell maintenance, expansion and/or differentiation, and influences cell localization. In certain embodiments, the hematopoietic growth factor that promotes hematopoietic cell maintenance, expansion and/or differentiation, and influences cell localization, may be an agent that includes interleukin 3, interleukin 6, interleukin 7, interleukin 11, interleukin 12, stem cell factor, FLK-2 ligand, FLT-2 ligand, Epo, Tpo, GMCSF, GCSF, Oncostatin M, and MCSF.

According to another aspect of the invention, a method for *in vivo* maintenance, expansion and/or differentiation of hematopoietic progenitor cells is provided. The method involves implanting into a subject a porous, solid matrix having seeded therein hematopoietic progenitor cells (which may include their progeny) and lymphoreticular stromal cells. The porous matrix has interconnected pores of a pore size sufficient to permit the cells to grow throughout the matrix and is an open cell porous matrix having a percent open space of at least 50%, and preferably at least 75%. Various embodiments are provided, wherein the porous solid matrix has one or more of the preferred characteristics as described above.

In certain embodiments, hematopoietic progenitor cells (that may include progeny) and lymphoreticular stromal cells are attached to the matrix by introducing *in vitro* an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into the porous solid matrix, and co-culturing the hematopoietic progenitor cells in an environment that is free of stromal cell conditioned medium and free of exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion and/or differentiation, other than serum. Various other embodiments are provided, wherein the co-culturing is performed under conditions as described above. In yet other embodiments, the porous solid matrix having seeded hematopoietic progenitor cells (that may include progeny) and lymphoreticular stromal cells is impregnated with a gelatinous agent that occupies pores of the matrix.

According to one aspect of the invention, a method for inducing T cell anergy *in vitro* is provided. The method involves introducing an amount of hematopoietic progenitor cells, an amount of antigen presenting cells, and an amount of lymphoreticular stromal cells into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the

matrix, and co-culturing the hematopoietic progenitor cells, the antigen presenting cells and the lymphoreticular stromal cells in the presence of at least one antigen under conditions sufficient to induce the formation of T cells and/or T cell progenitors and to inhibit activation of the formed T cells and/or T cell progenitors.

5 In certain embodiments, the hematopoietic progenitor cells may be pluripotent stem cells, multipotent progenitor cells and/or progenitor cells committed to specific hematopoietic lineages. The hematopoietic progenitor cells may be derived from a tissue such as bone marrow, peripheral blood (including mobilized peripheral blood), umbilical cord blood, placental blood, fetal liver, embryonic cells (including embryonic stem cells), aortal-gonadal-
10 mesonephros derived cells, and lymphoid soft tissue. Lymphoid soft tissue includes the thymus, spleen, liver, lymph node, skin, tonsil and/or Peyer's patches. In other embodiments, the lymphoreticular stromal cells may be also derived from at least one of the foregoing lymphoid soft tissues. In preferred embodiments, the lymphoreticular stromal cells are thymic stromal cells and the multipotent progenitor cells and/or committed progenitor cells
15 are committed to a T cell lineage. In other embodiments, the hematopoietic progenitor cells and/or the lymphoreticular stromal cells may be genetically altered. In certain embodiments, the antigen presenting cells include cells such as dendritic cells, monocytes/macrophages, Langerhans cells, Kupfer cells, microglia, alveolar macrophages and B cells. In other embodiments, the antigen presenting cells are derived from hematopoietic progenitor cells *in*
20 *vitro*. Various embodiments are provided, wherein the porous solid matrix has one or more of the preferred characteristics as described above.

According to another aspect of the invention, a method for inducing T cell reactivity *in vitro* is provided. The method involves introducing an amount of hematopoietic progenitor cells, an amount of antigen presenting cells, and an amount of lymphoreticular stromal cells
25 into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix, and co-culturing the hematopoietic progenitor cells, the antigen presenting cells and the lymphoreticular stromal cells in the presence of at least one antigen under conditions sufficient to induce the formation of T cells and/or T cell progenitors from the hematopoietic
30 progenitor cells having specificity for the at least one antigen. Various embodiments are provided, wherein the hematopoietic progenitor cells, the lymphoreticular stromal cells, and the porous solid matrix have one or more of the preferred characteristics as described above, and the cells are cultured as described above. In certain embodiments, the antigen presenting

cells include cells such as dendritic cells, monocytes/macrophages, Langerhans cells, Kupfer cells, microglia, alveolar macrophages and B cells. In other embodiments, the antigen presenting cells are derived from hematopoietic progenitor cells *in vitro*. In further
5 culture. Preferred co-stimulatory agents include lymphocyte function associated antigen 3 (LFA-3), CD2, CD40, CD80/B7-1, CD86/B7-2, OX-2, CD70, and CD82.

In yet another aspect of the invention, a solid porous matrix is provided wherein hematopoietic progenitor cells, with or without their progeny, and lymphoreticular stromal cells are attached to the solid porous matrix. The lymphoreticular stromal cells are present in
10 an amount sufficient to support the growth and differentiation of hematopoietic progenitor cells. In certain embodiments, the hematopoietic progenitor cells are attached to the lymphoreticular stromal cells. The porous matrix can be one that is an open cell porous matrix having a percent open space of at least 50%, and preferably at least 75%. In one embodiment the porous solid matrix has pores defined by interconnecting ligaments having a diameter at
15 midpoint, on average, of less than 150 μm . Preferably the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material, the metal coating being selected from the group consisting of tantalum, titanium, platinum (including other metals of the platinum group), niobium, hafnium, tungsten, and combinations thereof. In preferred
20 embodiments, whether the porous solid matrix is metal-coated or not, the matrix is coated with a biological agent selected from the group consisting of collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitrogen, antibodies and fragments thereof, functional equivalents of these factors, and combinations thereof. Most preferably the metal coating is tantalum coated with a biological agent. In
25 certain other embodiments the porous solid matrix having seeded hematopoietic progenitor cells and lymphoreticular stromal cells, is impregnated with a gelatinous agent that occupies pores of the matrix.

In a further aspect of the invention, a method for identifying an agent suspected of affecting hematopoietic cell development, is provided. The method involves introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into
30 a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix, co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells in the presence of at least one candidate agent suspected of affecting hematopoietic cell

development (in a test co-culture), and determining whether the at least one candidate agent affects hematopoietic cell development in the test co-culture by comparing the test co-culture hematopoietic cell development to a control co-culture, whereby hematopoietic progenitor cells and lymphoreticular stromal cells are co-cultured in the absence of the at least one candidate agent. Various embodiments are provided, wherein the hematopoietic progenitor cells, the lymphoreticular stromal cells, and the porous solid matrix have one or more of the preferred characteristics as described above, and the cells are cultured as described above. In certain embodiments, hematopoietic progenitor cell development includes hematopoietic progenitor cell maintenance, expansion, differentiation toward a specific cell lineage, and/or cell-death (including apoptosis). In preferred embodiments the lymphoreticular stromal cells are thymic stromal cells.

In another aspect of the invention, a method for isolating from a cell culture an agent suspected of affecting hematopoietic cell development, is provided. The method involves introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix, co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells, obtaining a test-supernatant from the co-culture, comparing the test-supernatant to a control-supernatant, and obtaining a subfraction of the test-supernatant that contains an agent suspected of affecting hematopoietic cell development that is absent from the control-supernatant. In certain embodiments the agent suspected of affecting hematopoietic cell development may be present in the control-supernatant and absent from the test-supernatant. In other embodiments, the agent suspected of affecting hematopoietic cell development in one supernatant may be different to an agent suspected of affecting hematopoietic cell development in the other supernatant (e.g., in size, via a post-translational modification, in an alternatively spliced variant form, etc.). Various embodiments are provided, wherein the hematopoietic progenitor cells, the lymphoreticular stromal cells, and the porous solid matrix have one or more of the preferred characteristics as described above, and the cells are cultured as described above. In certain embodiments, hematopoietic progenitor cell development includes hematopoietic progenitor cell maintenance, expansion, differentiation toward a specific cell lineage, and/or cell-death (including apoptosis). In preferred embodiments, the lymphoreticular stromal cells are thymic stromal cells. In certain other embodiments, the control culture system of the prior art

(where the control-supernatant can be obtained from) is the one described in U.S. Patent No. 5,677,139 by Johnson *et al.*

These and other aspects of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments.

Brief Description of the Drawings

Figure 1 shows the differentiation of human CD34⁺ progenitor cells into T cells, in co-culture with murine thymic stroma cells on a three-dimensional matrix; the data in Fig. 1(a) shows the acquisition of CD2 and the down-regulation of the hematopoietic progenitor cell marker CD34; the data in Fig. 1(b) shows the discrete populations of SP CD4⁺ and SP CD8⁺ cells, including their DP CD4⁺CD8⁺ precursors; the data in Figs. 1(c and d) shows that all CD4⁺(c) and CD8⁺(d) cells co-expressed CD3.

Figure 2 shows the intrasample variability in numbers of T cells generated in a co-culture system of the invention.

Figure 3 shows the intersample variability in numbers of T cells generated in a co-culture system of the invention.

Detailed Description of the Invention

The invention involves the unexpected discovery that hematopoietic progenitor cells co-cultured with lymphoreticular stromal cells in a porous solid scaffold, without the addition of exogenous growth agents, generate at a fast rate an unexpectedly high number of functional, differentiated progeny of a lymphoid tissue-specific lineage. Also surprising, according to the invention, has been the discovery that lesser amounts of nonlymphoid cells (i.e. myelo-monocytic cells) are generated from the co-culture of hematopoietic progenitor cells and lymphoreticular stromal cells in a porous solid scaffold of the invention when compared to existing technology. Thus, the present invention, and in contrast to what has been previously achieved in the art, permits for the rapid generation of a large number of differentiated, lymphoid-specific cells from a relatively small number of hematopoietic progenitor cells.

Methods of the invention are therefore useful *inter alia* for establishing immunocompetence in patients suffering from an immunodeficiency, e.g., a T cell or B cell deficiency, e.g., a thymic based immunodeficiency, e.g., a congenital immunodeficiency due to thymic aplasia or dysfunction, an acquired immune disorder, e.g., AIDS, immunoincompetence resulting from a neoplastic disease, or immunoincompetence resulting

from a medical procedure, e.g., chemotherapy, immunocompetence in response to an antigen, etc.

The invention in one aspect involves culturing hematopoietic cells in a porous solid matrix, in the absence of exogenous growth agents, to produce lymphoid tissue origin
5 (lymphoid tissue-specific) cells.

A porous, solid matrix, is defined as a three-dimensional structure with "sponge-like" continuous pores forming an interconnecting network. The matrix can be rigid or elastic, and it provides a scaffold upon which cells can grow throughout. Its pores are interconnected and provide the continuous network of channels extending through the matrix and also permit the
10 flow of nutrients throughout. A preferred matrix is an open cell foam matrix having a percent open space of at least 50% and preferably 75%. Thus, it is preferred that the open space comprise the majority of the matrix. This is believed to maximize cell migration, cell-cell contact, space for cell growth and accessibility to nutrients. It is preferred that the porous matrix be formed of a reticulated matrix of ligaments which at their center point are less than
15 150 μ m in diameter, preferably 60 μ m, whereby a cell can reside on or interact with a portion of the ligament. Preferably, the average pore diameter is on the order of 300 μ m, which resembles cancellous bone. Suitable matrices can be obtained using a number of different methods. Examples of such methods include solvent casting or extraction of polymers, track etching of a variety of materials, foaming of a polymer, the replamineform process for
20 hydroxyapatite, and other methodologies well known to those of ordinary skill in the art. The materials employed can be natural or synthetic, including biological materials such as proteins, hyaluronic acids, synthetic polymers such as polyvinyl pyrrolidones, polymethylmethacrylate, methyl cellulose, polystyrene, polypropylene, polyurethane, ceramics such as tricalcium phosphate, calcium aluminate, calcium hydroxyapatite and
25 ceramic-reinforced or coated polymers. If the starting material for the scaffold is not metal, a metal coating can be applied to the three-dimensional matrix. Metal coatings provide further structural support and/or cell growth and adhesive properties to the matrix. Preferred metals used as coatings comprise tantalum, titanium, platinum and metals in the same element group as platinum, niobium, hafnium, tungsten, and combinations of alloys thereof. Coating
30 methods for metals include a process such as CVD (Chemical Vapor Deposition).

The preferred matrix, referred to herein throughout as Cellfoam (Cytomatrix, Woburn, MA), is described in detail in U.S. Patent No. 5,282,861, and is incorporated herein by reference. More specifically, the preferred matrix is a reticulated open cell substrate formed

by a lightweight, substantially rigid foam of carbon-containing material having open spaces defined by an interconnecting network, wherein said foam material has interconnected continuous channels, and a thin film of metallic material deposited onto the reticulated open cell substrate and covering substantially all of the interconnecting network to form a composite porous biocompatible material creating a porous microstructure similar to that of natural cancellous bone.

Additionally, such matrices can be coated with biological agents which can promote cell adhesion for the cultured hematopoietic progenitor cells, allowing for improved migration, growth and proliferation. Moreover, when these matrices are used for the *in vivo* maintenance, expansion and/or differentiation of hematopoietic progenitor cells (i.e., when the matrices with the cells are implanted into a subject, -see also discussion below), biological agents that promote angiogenesis (vascularization) and biological agents that prevent/reduce inflammation may also be used for coating of the matrices. Preferred biological agents comprise collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitrogen, antibodies and fragments thereof, functional equivalents of these agents, and combinations thereof.

Angiogenic factors include platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), bFGF-2, leptins, plasminogen activators (tPA, uPA), angiopoietins, lipoprotein A, transforming growth factor- β , bradykinin, angiogenic oligosaccharides (e.g., hyaluronan, heparan sulphate), thrombospondin, hepatocyte growth factor (also known as scatter factor) and members of the CXC chemokine receptor family. Anti-inflammatory factors comprise steroidal and non-steroidal compounds and examples include: Alclofenac; Alclometasone Dipropionate; Algestone Acetonide; Alpha Amylase; Amcinafal; Amcinafide; Amfenac Sodium; Amiprilose Hydrochloride; Anakinra; Aniolac; Anitrazafen; Apazone; Balsalazide Disodium; Bendazac; Benoxaprofen; Benzydamine Hydrochloride; Bromelains; Broperamole; Budesonide; Carprofen; Cicloprofen; Cintazone; Cliprofen; Clobetasol Propionate; Clobetasone Butyrate; Clopirac; Cloticasone Propionate; Cormethasone Acetate; Cortodoxone; Deflazacort; Desonide; Desoximetasone; Dexamethasone Dipropionate; Diclofenac Potassium; Diclofenac Sodium; Diflorasone Diacetate; Diflumidone Sodium; Diflunisal; Difluprednate; Diftalone; Dimethyl Sulfoxide; Drocinonide; Endrysone; Enlimomab; Enolicam Sodium; Epirizole; Etodolac; Etofenamate; Felbinac; Fenamole; Fenbufen; Fenclofenac; Fenclorac; Fendosal; Fenpipalone; Fentiazac; Flazalone; Fluazacort; Flufenamic Acid; Flumizole; Flunisolid

Acetate; Flunixin; Flunixin Meglumine; Fluocortin Butyl; Fluorometholone Acetate;
 Fluquazone; Flurbiprofen; Fluretofen; Fluticasone Propionate; Furaprofen; Furobufen;
 Halcinonide; Halobetasol Propionate; Halopredone Acetate; Ibufenac; Ibuprofen; Ibuprofen
 Aluminum; Ibuprofen Piconol; Ilonidap; Indomethacin; Indomethacin Sodium; Indoprofen;
 5 Indoxole; Intrazole; Isoflupredone Acetate; Isoxepac; Isoxicam; Ketoprofen; Lofemizole
 Hydrochloride; Lornoxicam; Loteprednol Etabonate; Meclofenamate Sodium; Meclofenamic
 Acid; Meclorison Dibutyrate; Mefenamic Acid; Mesalamine; Meseclazone;
 Methylprednisolone Suleptanate; Morniflumate; Nabumetone; Naproxen; Naproxen Sodium;
 Naproxol; Nimazone; Olsalazine Sodium; Orgotein; Orpanoxin; Oxaprozin;
 10 Oxyphenbutazone; Paranyline Hydrochloride; Pentosan Polysulfate Sodium; Phenbutazone
 Sodium Glycerate; Pirfenidone; Piroxicam; Piroxicam Cinnamate; Piroxicam Olamine;
 Pirprofen; Prednazate; Prifelone; Prodolic Acid; Proquazone; Proxazole; Proxazole Citrate ;
 Rimexolone; Romazarit ; Salcolex ; Salnacedin; Salsalate ; Sanguinarium Chloride ;
 Seclazone ; Sermetacin; Sudoxicam; Sulindac; Suprofen; Talmetacin; Talniflumate;
 15 Talosalate; Tebufelone; Tenidap; Tenidap Sodium; Tenoxicam; Tesicam; Tesimide;
 Tetrydamine ; Tiopinac ; Tixocortol Pivalate; Tolmetin; Tolmetin Sodium; Triclonide;
 Triflumidate; Zidometacin; Zomepirac Sodium.

In certain embodiments of the invention the porous solid matrix having seeded
 hematopoietic progenitor cells, with or without their progeny, and lymphoreticular stromal
 20 cells is impregnated with a gelatinous agent that occupies pores of the matrix. The
 hematopoietic progenitor cells, with or without their progeny, and/or the lymphoreticular
 stromal cells can be seeded prior to, substantially at the same time as, or following
 impregnation (or infiltration) with a gelatinous agent. For example, the cells may be mixed
 with the agent and seeded at the same time as the the impregnation of the matrix with the
 25 agent. In some embodiments, the cells are seeded onto the porous solid matrix prior to
 application of the agent. In certain embodiments the lymphoreticular stromal cells are seeded
 in a similar manner. A person of ordinary skill in the art can easily determine seeding
 conditions. Preferably the lymphoreticular stromal cells are seeded prior to the hematopoietic
 progenitor cells and prior to impregnation with the agent.

30 "Impregnation" with a gelatinous agent can serve, *inter alia*, to contain the cells
 within the matrix, or to help maintain and/or enhance cell attachment onto the matrix. The
 "gelatinous" agent may be one that can be maintained in a fluid state initially (i.e. gelable),
 and after its application into the matrix, be gelatinized *in situ* in the matrix. Such

gelatinization may occur in a number of different ways, including altering the agent's temperature, irradiating the agent with an energy source (e.g., light), etc. The "gelatinous" agent also is characterized by its ability to allow the nutrients of the growth media to reach the cells throughout the matrix. Exemplary "gelatinous" agents include cellulosic polysaccharides (such as cellulose, hemicellulose, methylcellulose, and the like), agar, agarose, albumin, algal mucin, mucin, mucilage, collagens, glycosaminoglycans, and proteoglycans (including their sulphated forms). In certain embodiments, the gelatinous agent may impregnate the matrix completely, in some embodiments partially, and in other embodiments minimally, serving only as a coating of all or some of the outer surfaces of the matrix. In important embodiments where gelatinous agents are employed, the "gelatinous" agent is methylcellulose and the impregnation is complete.

According to the invention, hematopoietic progenitor cells and lymphoreticular stromal cells are co-cultured in one of the foregoing porous solid matrices, in the absence of exogenous growth agents, to produce lymphoid tissue origin (lymphoid tissue-specific) cells. "Lymphoid tissue origin" (lymphoid tissue-specific) cells, as used herein, refer to cells that may be produced *in vitro* or *in vivo* according to the invention, and are substantially similar (e.g., in properties and function) to the cells produced naturally *in vivo* from organs and tissues that include the bone marrow, thymus, lymph nodes, spleen and mucosal associated lymphoid tissue (unencapsulated tissue lining the respiratory, alimentary and genito-urinary tracts).

"Hematopoietic progenitor cells" as used herein refers to immature blood cells having the capacity to self-renew and to differentiate into the more mature blood cells (also described herein as "progeny") comprising granulocytes (e.g., promyelocytes, neutrophils, eosinophils, basophils), erythrocytes (e.g., reticulocytes, erythrocytes), thrombocytes (e.g., megakaryoblasts, platelet producing megakaryocytes, platelets), and monocytes (e.g., monocytes, macrophages). It is known in the art that such cells may or may not include CD34⁺ cells. CD34⁺ cells are immature cells present in the "blood products" described below, express the CD34 cell surface marker, and are believed to include a subpopulation of cells with the "progenitor cell" properties defined above. It is well known in the art that hematopoietic progenitor cells include pluripotent stem cells, multipotent progenitor cells (e.g., a lymphoid stem cell), and/or progenitor cells committed to specific hematopoietic lineages. The progenitor cells committed to specific hematopoietic lineages may be of T cell

lineage, B cell lineage, dendritic cell lineage, Langerhans cell lineage and/or lymphoid tissue-specific macrophage cell lineage.

The hematopoietic progenitor cells can be obtained from blood products. A “blood product” as used in the present invention defines a product obtained from the body or an organ of the body containing cells of hematopoietic origin. Such sources include unfractionated bone marrow, umbilical cord, peripheral blood, liver, thymus, lymph and spleen. It will be apparent to those of ordinary skill in the art that all of the aforementioned crude or unfractionated blood products can be enriched for cells having “hematopoietic progenitor cell” characteristics in a number of ways. For example, the blood product can be depleted from the more differentiated progeny. The more mature, differentiated cells can be selected against, via cell surface molecules they express. Additionally, the blood product can be fractionated selecting for CD34⁺ cells. As mentioned earlier, CD34⁺ cells are thought in the art to include a subpopulation of cells capable of self-renewal and pluripotentiality. Such selection can be accomplished using, for example, commercially available magnetic anti-CD34 beads (Dynal, Lake Success, NY). Unfractionated blood products can be obtained directly from a donor or retrieved from cryopreservative storage.

The cells co-cultured with the hematopoietic progenitor cells according to the methods of the invention are lymphoreticular stromal cells. “Lymphoreticular stromal cells” as used herein may include, but are not limited to, all cell types present in a lymphoid tissue which are not lymphocytes or lymphocyte precursors or progenitors, e.g., epithelial cells, endothelial cells, mesothelial cells, dendritic cells, splenocytes and macrophages. Lymphoreticular stromal cells also include cells that would not ordinarily function as lymphoreticular stromal cells, such as fibroblasts, which have been genetically altered to secrete or express on their cell surface the factors necessary for the maintenance, growth and/or differentiation of hematopoietic progenitor cells, including their progeny. Lymphoreticular stromal cells are derived from the disaggregation of a piece of lymphoid tissue (see discussion below and the Examples). Such cells according to the invention are capable of supporting *in vitro* the maintenance, growth and/or differentiation of hematopoietic progenitor cells, including their progeny. By “lymphoid tissue” it is meant to include bone marrow, peripheral blood (including mobilized peripheral blood), umbilical cord blood, placental blood, fetal liver, embryonic cells (including embryonic stem cells), aortal-gonadal-mesonephros derived cells, and lymphoid soft tissue. “Lymphoid soft tissue” as used herein includes, but is not limited

to, tissues such as thymus, spleen, liver, lymph node, skin, tonsil, adenoids and Peyer's patch, and combinations thereof.

Lymphoreticular stromal cells provide the supporting microenvironment in the intact lymphoid tissue for the maintenance, growth and/or differentiation of hematopoietic progenitor cells, including their progeny. The microenvironment includes soluble and cell surface factors expressed by the various cell types which comprise the lymphoreticular stroma. Generally, the support which the lymphoreticular stromal cells provide may be characterized as both contact-dependent and non-contact-dependent.

Lymphoreticular stromal cells may be allogeneic, syngeneic or xenogeneic with respect to the hematopoietic progenitor cells. Lymphoreticular stroma cells may be obtained from the lymphoid tissue of a human or a non-human subject at any time after the organ/tissue has developed to a stage (i.e., the maturation stage) at which it can support the maintenance growth and/or differentiation of hematopoietic progenitor cells. The stage will vary between organs/tissues and between subjects. In primates, for example, the maturation stage of thymic development is achieved during the second trimester. At this stage of development the thymus can produce peptide hormones such as thymulin, α_1 and β_4 -thymosin, and thymopoietin, as well as other factors required to provide the proper microenvironment for T cell differentiation. The different maturation stages for the different organs/tissues and between different subjects are well known in the art.

The lymphoid tissue from which lymphoreticular stromal cells are derived usually determines the lineage-commitment hematopoietic progenitor cells undertake, resulting in the lineage-specificity of the differentiated progeny. In certain embodiments, the lymphoreticular stromal cells are thymic stromal cells and the multipotent progenitor cells and/or committed progenitor cells are committed to a T cell lineage. In other embodiments, the lymphoreticular stromal cells may be splenic stromal cells and the multipotent progenitor cells and/or committed progenitor cells are committed to a B cell lineage. Also surprising, according to the invention, has been the discovery that the highest yield of differentiated progeny occurs when human hematopoietic progenitor cells are cultured in the presence of xenogeneic (non-human) lymphoreticular stromal cells. Preferably the xenogeneic lymphoreticular stromal cells are of murine origin.

Unexpectedly, it has also been discovered that lesser amounts of nonlymphoid-specific cells (i.e. myelo-monocytic cells) are generated from the foregoing co-cultures when compared to existing methodology. In other words, more homogeneous differentiation of

cells with fewer contaminant cell types (nonlymphoid) is observed from cultures of the present invention on Cellfoam, enabling the preservation of immature progenitors (CD34⁺ cells) while promoting the differentiation of more mature T progeny.

Various other embodiments are provided, wherein the lymphoreticular stromal cells may be genetically altered. The lymphoreticular stromal cells may be transfected with exogenous DNA that encodes, for example, one of the hematopoietic growth factors described above (see fibroblast discussion above).

As mentioned earlier, lymphoreticular stromal cells are derived from the disaggregation of a piece of lymphoid tissue, forming cell suspensions. Preferably, single cell suspensions are generated. These lymphoreticular stromal cell suspensions may be used directly, or made non-mitotic by procedures standard in the tissue culture art. Examples of such methods are irradiation of lymphoreticular stromal cells with a gamma-ray source or incubation of the cells with mitomycin C for a sufficient amount of time to render the cells mitotically inactive. Mitotic inactivation is preferred when the lymphoreticular stromal cells are of human origin (to eliminate progenitor cells that may be present in the suspension). The lymphoreticular stromal cells may then be seeded into a three-dimensional matrix of the invention and permitted to attach to a surface of the porous, solid matrix. It should be noted that the lymphoreticular stromal cells may alternatively be cryopreserved for later use or for storage and shipment to remote locations, such as for use in connection with the sale of kits. Cryopreservation of cells cultured *in vitro* is well established in the art. Subsequent to isolation (and/or mitotic inactivation) of a cell sample, cells may be cryopreserved by first suspending the cells in a cryopreservation medium and then gradually freezing the cell suspension. Frozen cells are typically stored in liquid nitrogen or at an equivalent temperature in a medium containing serum and a cryopreservative such as dimethyl sulfoxide.

The co-culture of the hematopoietic progenitor cells (and progeny thereof) with lymphoreticular stromal cells, preferably occurs under conditions sufficient to produce a percent increase in the number of lymphoid tissue origin cells deriving from the hematopoietic progenitor cells. The conditions used refer to a combination of conditions known in the art (e.g., temperature, CO₂ and O₂ content, nutritive media, time-length, etc.). The time sufficient to increase the number of cells is a time that can be easily determined by a person skilled in the art, and can vary depending upon the original number of cells seeded. The amounts of hematopoietic progenitor cells and lymphoreticular stromal cells initially introduced (and subsequently seeded) into the porous solid matrix may vary according to the

needs of the experiment. The ideal amounts can be easily determined by a person skilled in the art in accordance with needs. Preferably, the lymphoreticular stromal cells would form a confluent layer onto the matrix. Hematopoietic progenitor cells may be added at different numbers. As an example, discoloration of the media over a certain period of time can be used
5 as an indicator of confluency. Additionally, and more precisely, different numbers of hematopoietic progenitor cells or volumes of the blood product can be cultured under identical conditions, and cells can be harvested and counted over regular time intervals, thus generating the "control curves". These "control curves" can be used to estimate cell numbers in subsequent occasions (see the Examples section).

10 The conditions for determining colony forming potential are similarly determined. Colony forming potential is the ability of a cell to form progeny. Assays for this are well known to those of ordinary skill in the art and include seeding cells into a semi-solid matrix, treating them with growth factors, and counting the number of colonies.

In preferred embodiments of the invention, the hematopoietic progenitor cells may be
15 harvested. "Harvesting" hematopoietic progenitor cells is defined as the dislodging or separation of cells from the matrix. This can be accomplished using a number of methods, such as enzymatic and non-enzymatic, centrifugal, electrical or by size, or the one preferred in the present invention, by flushing of the cells using the media in which the cells are incubated. The cells can be further collected, separated, and further expanded generating even larger
20 populations of differentiated progeny.

As mentioned above, the hematopoietic progenitor cells, and progeny thereof, can be genetically altered. Genetic alteration of a hematopoietic progenitor cell includes all transient and stable changes of the cellular genetic material which are created by the addition of exogenous genetic material. Examples of genetic alterations include any gene therapy
25 procedure, such as introduction of a functional gene to replace a mutated or nonexpressed gene, introduction of a vector that encodes a dominant negative gene product, introduction of a vector engineered to express a ribozyme and introduction of a gene that encodes a therapeutic gene product. Natural genetic changes such as the spontaneous rearrangement of a T cell receptor gene without the introduction of any agents are not included in this concept.
30 Exogenous genetic material includes nucleic acids or oligonucleotides, either natural or synthetic, that are introduced into the hematopoietic progenitor cells. The exogenous genetic material may be a copy of that which is naturally present in the cells, or it may not be

naturally found in the cells. It typically is at least a portion of a naturally occurring gene which has been placed under operable control of a promoter in a vector construct.

The invention involves the unexpected discovery that hematopoietic progenitor cells can be more efficiently genetically altered if the genetic alteration occurs while the hematopoietic progenitor cells are on and within a solid porous matrix as described above.

Various techniques may be employed for introducing nucleic acids into cells. Such techniques include transfection of nucleic acid-CaPO₄ precipitates, transfection of nucleic acids associated with DEAE, transfection with a retrovirus including the nucleic acid of interest, liposome mediated transfection, and the like. For certain uses, it is preferred to target the nucleic acid to particular cells. In such instances, a vehicle used for delivering a nucleic acid according to the invention into a cell (e.g., a retrovirus, or other virus; a liposome) can have a targeting molecule attached thereto. For example, a molecule such as an antibody specific for a surface membrane protein on the target cell or a ligand for a receptor on the target cell can be bound to or incorporated within the nucleic acid delivery vehicle. For example, where liposomes are employed to deliver the nucleic acids of the invention, proteins which bind to a surface membrane protein associated with endocytosis may be incorporated into the liposome formulation for targeting and/or to facilitate uptake. Such proteins include proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half life, and the like. Polymeric delivery systems also have been used successfully to deliver nucleic acids into cells, as is known by those skilled in the art. Such systems even permit oral delivery of nucleic acids.

In the present invention, the preferred method of introducing exogenous genetic material into hematopoietic cells is by transducing the cells *in situ* on the matrix using replication-deficient retroviruses. Replication-deficient retroviruses are capable of directing synthesis of all virion proteins, but are incapable of making infectious particles. Accordingly, these genetically altered retroviral vectors have general utility for high-efficiency transduction of genes in cultured cells, and specific utility for use in the method of the present invention. Retroviruses have been used extensively for transferring genetic material into cells. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral

particles from tissue culture media, and infection of the target cells with the viral particles) are provided in the art.

The major advantage of using retroviruses is that the viruses insert efficiently a single copy of the gene encoding the therapeutic agent into the host cell genome, thereby permitting the exogenous genetic material to be passed on to the progeny of the cell when it divides. In addition, gene promoter sequences in the LTR region have been reported to enhance expression of an inserted coding sequence in a variety of cell types. The major disadvantages of using a retrovirus expression vector are (1) insertional mutagenesis, i.e., the insertion of the therapeutic gene into an undesirable position in the target cell genome which, for example, leads to unregulated cell growth and (2) the need for target cell proliferation in order for the therapeutic gene carried by the vector to be integrated into the target genome. Despite these apparent limitations, delivery of a therapeutically effective amount of a therapeutic agent via a retrovirus can be efficacious if the efficiency of transduction is high and/or the number of target cells available for transduction is high.

Yet another viral candidate useful as an expression vector for transformation of hematopoietic cells is the adenovirus, a double-stranded DNA virus. Like the retrovirus, the adenovirus genome is adaptable for use as an expression vector for gene transduction, i.e., by removing the genetic information that controls production of the virus itself. Because the adenovirus functions usually in an extrachromosomal fashion, the recombinant adenovirus does not have the theoretical problem of insertional mutagenesis. On the other hand, adenoviral transformation of a target hematopoietic cell may not result in stable transduction. However, more recently it has been reported that certain adenoviral sequences confer intrachromosomal integration specificity to carrier sequences, and thus result in a stable transduction of the exogenous genetic material.

Thus, as will be apparent to one of ordinary skill in the art, a variety of suitable vectors are available for transferring exogenous genetic material into hematopoietic cells. The selection of an appropriate vector to deliver a therapeutic agent for a particular condition amenable to gene replacement therapy and the optimization of the conditions for insertion of the selected expression vector into the cell, are within the scope of one of ordinary skill in the art without the need for undue experimentation. The promoter characteristically has a specific nucleotide sequence necessary to initiate transcription. Optionally, the exogenous genetic material further includes additional sequences (i.e., enhancers) required to obtain the desired gene transcription activity. For the purpose of this discussion an "enhancer" is simply any

nontranslated DNA sequence which works contiguous with the coding sequence (in cis) to change the basal transcription level dictated by the promoter. Preferably, the exogenous genetic material is introduced into the hematopoietic cell genome immediately downstream from the promoter so that the promoter and coding sequence are operatively linked so as to permit transcription of the coding sequence. A preferred retroviral expression vector includes an exogenous promoter element to control transcription of the inserted exogenous gene. Such exogenous promoters include both constitutive and inducible promoters.

Naturally-occurring constitutive promoters control the expression of essential cell functions. As a result, a gene under the control of a constitutive promoter is expressed under all conditions of cell growth. Exemplary constitutive promoters include the promoters for the following genes which encode certain constitutive or "housekeeping" functions: hypoxanthine phosphoribosyl transferase (HPRT), dihydrofolate reductase (DHFR) (Scharfmann et al., Proc. Natl. Acad. Sci. USA 88:4626-4630 (1991)), adenosine deaminase, phosphoglycerol kinase (PGK), pyruvate kinase, phosphoglycerol mutase, the actin promoter (Lai et al., Proc. Natl. Acad. Sci. USA 86: 10006-10010 (1989)), and other constitutive promoters known to those of skill in the art. In addition, many viral promoters function constitutively in eukaryotic cells. These include: the early and late promoters of SV40; the long terminal repeats (LTRS) of Moloney Leukemia Virus and other retroviruses; and the thymidine kinase promoter of Herpes Simplex Virus, among many others. Accordingly, any of the above-referenced constitutive promoters can be used to control transcription of a heterologous gene insert.

Genes that are under the control of inducible promoters are expressed only or to a greater degree, in the presence of an inducing agent, (e.g., transcription under control of the metallothionein promoter is greatly increased in presence of certain metal ions). Inducible promoters include responsive elements (REs) which stimulate transcription when their inducing factors are bound. For example, there are REs for serum factors, steroid hormones, retinoic acid and cyclic AMP. Promoters containing a particular RE can be chosen in order to obtain an inducible response and in some cases, the RE itself may be attached to a different promoter, thereby conferring inducibility to the recombinant gene. Thus, by selecting the appropriate promoter (constitutive versus inducible; strong versus weak), it is possible to control both the existence and level of expression of a therapeutic agent in the genetically modified hematopoietic cell. Selection and optimization of these factors for delivery of a therapeutically effective dose of a particular therapeutic agent is deemed to be within the

scope of one of ordinary skill in the art without undue experimentation, taking into account the above-disclosed factors and the clinical profile of the patient.

In addition to at least one promoter and at least one heterologous nucleic acid encoding the therapeutic agent, the expression vector preferably includes a selection gene, for example, a neomycin resistance gene, for facilitating selection of hematopoietic cells that have been transfected or transduced with the expression vector. Alternatively, the hematopoietic cells are transfected with two or more expression vectors, at least one vector containing the gene(s) encoding the therapeutic agent(s), the other vector containing a selection gene. The selection of a suitable promoter, enhancer, selection gene and/or signal sequence (described below) is deemed to be within the scope of one of ordinary skill in the art without undue experimentation.

The selection and optimization of a particular expression vector for expressing a specific gene product in an isolated hematopoietic cell is accomplished by obtaining the gene, preferably with one or more appropriate control regions (e.g., promoter, insertion sequence); preparing a vector construct comprising the vector into which is inserted the gene; transfecting or transducing cultured hematopoietic cells *in vitro* with the vector construct; and determining whether the gene product is present in the cultured cells.

Table 1. Human Gene Therapy Protocols Approved by RAC: 1990-1994

Severe combined immune deficiency (SCID) due to ADA deficiency	Autologous lymphocytes transduced with human ADA gene	7/31/90
Advanced cancer	Tumor-infiltrating lymphocytes transduced with tumor necrosis factor gene	7/31/90
Advanced cancer	Immunization with autologous cancer cells transduced with tumor necrosis factor gene	10/07/91
Advanced cancer	Immunization with autologous cancer cells transduced with interleukin-2 gene	10/07/91
Asymptomatic patients infected with HIV-1	Murine Retro viral vector encoding HIV-1 genes [HIV-IT(V)]	6/07/93
AIDS	Effects of a transdominant form of <i>rev</i> gene on AIDS intervention	6/07/93
Advanced cancer	Human multiple-drug resistance (MDR) gene transfer	6/08/93
HIV infection	Autologous lymphocytes transduced with catalytic ribozyme that cleaves HIV-1 RNA (Phase I study)	9/10/93
Metastatic melanoma	Genetically engineered autologous tumor vaccines producing interleukin-2	9/10/93
HIV infection	Murine Retro viral vector encoding HIV-IT(V) genes (open label Phase I/II trial)	12/03/93
HIV infection (identical twins)	Adoptive transfer of syngeneic cytotoxic T lymphocytes (Phase I/II pilot study)	3/03/94

Breast cancer (chemo-protection during therapy)	Use of modified Retro virus to introduce chemotherapy resistance sequences into normal hematopoietic cells (pilot study)	6/09/94
Fanconi's anemia	Retro viral mediated gene transfer of the Fanconi anemia complementation group C gene to hematopoietic progenitors	6/09/94
Metastatic prostate carcinoma	Autologous human granulocyte macrophage-colony stimulating factor gene transduced prostate cancer vaccine *(first protocol to be approved under the accelerated review process; ORDA=Office of Recombinate DNA Activities)	ORDA/NIH 8/03/94*
Metastatic breast cancer	<i>In vivo</i> infection with breast-targeted Retro viral vector expressing antisense <i>c-fos</i> or antisense <i>c-myc</i> RNA	9/12/94
Metastatic breast cancer (refractory or recurrent)	Non-viral system (liposome-based) for delivering human interleukin-2 gene into autologous tumor cells (pilot study)	9/12/94
Mild Hunter syndrome	Retro viral-mediated transfer of the iduronate-2-sulfatase gene into lymphocytes	9/13/94
Advanced mesothelioma	Use of recombinant adenovirus (Phase I study)	9/13/94

The foregoing (Table 1), represent only examples of genes that can be delivered according to the methods of the invention. Suitable promoters, enhancers, vectors, etc., for such genes are published in the literature associated with the foregoing trials. In general, useful genes replace or supplement function, including genes encoding missing enzymes such as adenosine deaminase (ADA) which has been used in clinical trials to treat ADA deficiency and cofactors such as insulin and coagulation factor VIII. Genes which affect regulation can also be administered, alone or in combination with a gene supplementing or replacing a specific function. For example, a gene encoding a protein which suppresses expression of a particular protein-encoding gene can be administered. The invention is particularly useful in delivering genes which stimulate the immune response, including genes encoding viral antigens, tumor antigens, cytokines (e.g. tumor necrosis factor) and inducers of cytokines (e.g. endotoxin).

Employing the culture conditions described in greater detail below, it is possible according to the invention to preserve hematopoietic progenitor cells and to stimulate the expansion of hematopoietic progenitor cell number and/or colony forming unit potential. Once expanded, the cells, for example, can be returned to the body to supplement, replenish, etc. a patient's hematopoietic progenitor cell population. This might be appropriate, for example, after an individual has undergone chemotherapy. There are certain genetic conditions wherein hematopoietic progenitor cell numbers are decreased, and the methods of the invention may be used in these situations as well.

It also is possible to take the increased numbers of hematopoietic progenitor cells produced according to the invention and stimulate them with hematopoietic growth agents that promote hematopoietic cell maintenance, expansion and/or differentiation, and also influence cell localization, to yield the more mature blood cells, *in vitro*. Such expanded populations of blood cells may be applied *in vivo* as described above, or may be used experimentally as will be recognized by those of ordinary skill in the art. Such differentiated cells include those described above, as well as T cells, plasma cells, erythrocytes, megakaryocytes, basophils, polymorphonuclear leukocytes, monocytes, macrophages, eosinophils and platelets.

In all of the culturing methods according to the invention, except as otherwise provided, the media used is that which is conventional for culturing cells. Examples include RPMI, DMEM, Iscove's, etc. Typically these media are supplemented with human or animal plasma or serum. Such plasma or serum can contain small amounts of hematopoietic growth factors. The media used according to the present invention, however, can depart from that used conventionally in the prior art. In particular, it has been discovered, surprisingly, that hematopoietic progenitor cells can be cultured on the matrices described above for extended periods of time without the need for adding any exogenous growth agents (other than those which may be contained in plasma or serum, hereinafter "serum"), without inoculating the environment of the culture with stromal cells and without using stromal cell conditioned media. Prior to the present invention, at least one of the foregoing agents was believed necessary in order to culture hematopoietic progenitor cells.

The growth agents of particular interest in connection with the present invention are hematopoietic growth factors. By hematopoietic growth factors, it is meant factors that influence the survival, proliferation or differentiation of hematopoietic progenitor cells. Growth agents that affect only survival and proliferation, but are not believed to promote differentiation, include the interleukins 3, 6 and 11, stem cell factor and FLT-3 ligand. Hematopoietic growth factors that promote differentiation include the colony stimulating factors such as GM-CSF, G-CSF, M-CSF, Tpo, Epo, Oncostatin M, and interleukins other than IL-3, 6 and 11. The foregoing factors are well known to those of ordinary skill in the art. Most are commercially available. They can be obtained by purification, by recombinant methodologies or can be derived or synthesized synthetically.

"Stromal cell conditioned medium" refers to medium in which the aforementioned lymphoreticular stromal cells have been incubated. The incubation is performed for a period

sufficient to allow the stromal cells to secrete factors into the medium. Such "stromal cell conditioned medium" can then be used to supplement the culture of hematopoietic progenitor cells promoting their proliferation and/or differentiation.

Thus, when cells are cultured without any of the foregoing agents, it is meant herein
5 that the cells are cultured without the addition of such agent except as may be present in serum, ordinary nutritive media or within the blood product isolate, unfractionated or fractionated, which contains the hematopoietic progenitor cells.

According to another aspect of the invention a method for *in vivo* maintenance,
expansion and/or differentiation of hematopoietic progenitor cells is provided. The method
10 involves implanting into a subject a porous solid matrix having seeded hematopoietic progenitor cells, hematopoietic progenitor cell progeny, and lymphoreticular stromal cells. Implantation of matrices similar to the matrices of the invention is well known in the art (Stackpool, GJ, et al, Combined Orthopaedic Research Societies Meeting, Nov. 6-8, 1995, San Diego, CA, Abstract Book p. 45; Turner, TM, et al., 21st Annual Meeting of the Society
15 for Biomaterials, March 18-22, San Francisco, CA, Abstract Book p. 125). Such matrices are biocompatible (i.e., no immune reactivity-no rejection) and can be implanted and transplanted in a number of different tissues of a subject. Such methods are useful in a variety of ways, including the study of hematopoietic progenitor cell maintenance, expansion, differentiation and/or localization *in vivo*, in a number of different tissues of a subject, and/or between
20 different subjects.

As used herein, a subject is a human, non-human primate, cow, horse, pig, sheep, goat, dog, cat or rodent. Human hematopoietic progenitor cells and human subjects are particularly important embodiments. As described above, when the matrices of the invention are used for such *in vivo* implantation studies, biological agents that promote angiogenesis
25 (vascularization) and/or prevent/reduce inflammation may also be used for coating of the matrices. Preferred biological agents are as described above. Also as described above, the hematopoietic progenitor cells are pre-seeded onto the porous solid matrix and cultured *in vitro* according to the invention, before implantation into a subject. According to the invention, an amount of the cells is introduced *in vitro* into the porous solid matrix, and co-
30 cultured with lymphoreticular stromal cells in an environment that is free stromal cell conditioned medium, and exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion and/or differentiation, other than serum. Implantation is then carried out. In certain embodiments, stromal cell conditioned medium

and exogenous hematopoietic growth factors may be added during the *in vitro* culture before implantation.

According to one aspect of the invention, a method for inducing T cell reactivity/activation, *in vitro*, is provided. Induction of T cell reactivity/activation involves co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells in the presence of antigen presenting cells and an antigen, in one of the foregoing matrices, under conditions sufficient to induce the formation of T cells and/or T cell progenitors from the hematopoietic progenitor cells having specificity for the antigen. The foregoing conditions could easily be established by a person of ordinary skill in the art, without undue experimentation (see also Sprent J, et al., *J Immunother*, 1998, 21(3):181-187; Berridge MJ, *Crit Rev Immunol*, 1997, 17(2):155-178; Owen MJ, et al., *Curr Opin Immunol*, 1996, 8(2):191-198; Whitfield JF, et al., *Mol Cell Biochem*, 1979, 27(3):155-179; Fauci AS, et al., *Ann Intern Med*, 1983, 99(1):61-75). Antigen stimulation of T cells in the presence of APCs, induces an antigen specific response that can be measured using a proliferation assay or just by measuring IL-2 production (see discussion below). These cells can be detected by culturing T cells with antigen at an appropriate concentration (e.g., 0.1-1.0 μ M tetanus toxoid) in the presence of APCs. If antigen specific T cells are present they can be detected using the assays described below under self-tolerance/anergy. Stimulation of T cells in the presence of APCs may include co-stimulation with a co-stimulatory agent. Co-stimulatory agents include lymphocyte function associated antigen- 3 (LFA-3), CD2, CD40, CD80/B7-1, CD86/B7-2, OX-2, CD70, and CD82. Co-stimulatory agents may also be used in lieu of APCs, provided that MHC class II molecules and anti-CD3 antibodies are co-administered with the co-stimulatory agent(s).

One or more antigens can be used at the same time for incubation in the foregoing culture system. Preferably, the lymphoreticular stromal cells are thymic stromal cells and of murine origin when the hematopoietic progenitor cells being expanded are human. Therefore, large numbers of antigen-specific mature T and immature T cells may be obtained in a short period of time that were never before realized using existing art methodologies. The present invention thus becomes useful in a wide range of applications, including pre-exposure vaccination of individuals with *in vitro* primed T cells, treatment of cancer patients using tumor-targeted T cell immunotherapy, treatment of bone marrow transplant patients (for whom opportunistic infections, such as CMV, are problematic and yet amenable to treatment with targeted T cells such as CMV-targeted cytotoxic lymphocytes), enhancement of

conventional vaccination efficacy through T cell adjuvant therapy, treatment of outbreaks of emergent or re-emergent pathogens, etc. The antigen presenting cells include cells such as dendritic cells, monocytes/macrophages, Langerhans cells, Kupfer cells, microglia, alveolar macrophages and B cells, and methods for their isolation are well known in the art. The antigen presenting cells may also be derived from hematopoietic progenitor cells *in vitro*.

Immunological tolerance refers to the inhibition of a subject's ability to mount an immune response, e.g., to a donor antigen, which would otherwise occur in response to the introduction of a non-self antigen into the subject. Tolerance can involve humoral, cellular, or both humoral and cellular responses. Thymic education results in the generation of T cells capable of responding to a myriad of foreign antigens in the context of *self*-MHC, but not *self*-antigens alone. This is achieved primarily by a systematic rescue of appropriate thymocytes from programmed cell death, based on a theme of *self*-restriction, and the release of these cells into the periphery to serve as self-tolerant T cells.

Self-tolerance can be established *in vitro* under conditions known in the art that include coculturing CD34⁺ T progenitors derived from a donor (A), in the presence of thymic stroma from another individual (B). Briefly, thymic stroma is established from freshly isolated thymic tissue that is digested into a single cell suspension using a collagenase (20 µg/ml, Sigma Chemical Co.). Thymic stromal cultures are established by plating the cell suspension in 24 well plates at a concentration of 4 x 10⁶ viable cells per well in a volume of 2 ml R10 (RPMI plus 10%FCS). Cultures are incubated in a standard humidified tissue culture incubator at 37° C with 5% CO₂. After one to two days, non-adherent cells are removed by washing three times with R10. The stroma requires an additional 7-10 days to become confluent. The stroma is maintained in R10 which is changed at least twice per week. After 7-10 days in culture, CD34⁺ cells in R10 are added to the stroma at a concentration of 1-3 x 10⁵ cells per well. Cultures are fed bi-weekly using partial medium exchanges with R10 with no exogenous cytokines added to these cultures. After 14-21 days, the non-adherent cells are removed from the cultures. The remaining, attached cells are self-tolerant T cells that have developed *in vitro*.

Methods for determining if tolerance has been established *in vitro* are also known to a person of ordinary skill in the art, and involve measurement of a proliferative response to: *self* (A), as well as to the thymus donor (B), and a third party (C), peripheral blood mononuclear

cells (PBMCs). Briefly, PBMCs from A, B and C are prepared by Ficoll gradient centrifugation.

1 x 10⁵ responder cells (*in vitro* generated T cells from A) are plated out in multiple replicates in a 96 well plate. Stimulator cells (PBMCs from A, B and C) are irradiated (3000 Rads) and added in 12 replicates at 1 x 10⁵ cells per well. Con-A (5 µg/ml) is used as a positive control. After 4 days 1 µCi of ³H-Thymidine is added to each well, and the plates harvested 18-24 hours later. If tolerance has been established, the *in vitro* generated T cells will respond and proliferate when mixed with an unrelated third party (C), but do not proliferate when mixed with PBMCs from self (A) or the thymic donor (B).

According to another aspect of the invention, a method for inducing T cell anergy, *in vitro*, is provided. Induction of T cell anergy involves co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells in one of the foregoing matrices, in the presence of antigen under conditions sufficient to induce the formation of T cells and/or T cell progenitors and to inhibit activation of the formed T cells and/or T cell progenitors.

Anergy is defined as an unresponsive state of T cells (that is they fail to produce IL-2 on restimulation, or proliferate when restimulated)(Zamoyska R, *Curr Opin Immunol*, 1998, 10(1):82-87; Van Parijs L, et al., *Science*, 1998, 280(5361):243-248; Schwartz RH, *Curr Opin Immunol*, 1997, 9(3):351-357; Immunol Rev, 1993, 133:151-76). Anergy may, however, be irreversible. Anergy may be induced via antigen-specific T cell stimulation in the absence of co-stimulation (one signal vs. two signal hypothesis). Alternatively peptides of low affinity or very high concentrations of peptide even in the presence of co-stimulation can induce anergy. Anergy can be induced *in vitro* by culturing T cells in the absence of antigen presenting cells (B cells, macrophages or dendritic cells). These T cells are then exposed to antigen for example tetanus toxoid (e.g., 0.1-1.0 µM). An aliquot of the T cells is used to present antigen. This constitutes antigen presentation without co-stimulation and will induce anergy (Nelson A, et al., *In Immuno*, 1998, 10(9):1335-46). Alternatively T cells can be cocultured with APCs, in the context of very high (10-100 µM) or very low (0.01-0.05 µM) tetanus toxoid, which will induce a state of unresponsiveness.

Anergy can be measured by taking the T cells described above, and restimulating them with antigen (e.g., 0.1-1.0 µM tetanus toxoid) in the presence of APCs. If the cells are anergic they will not respond to antigen at an appropriate concentration in the context of APCs. Anergy is measured by culturing the cells as such for 3-5 days and measuring proliferation or

the lack thereof as follows. Briefly APCs are plated out in multiple replicates in a 96 well plate, after irradiation (3000 Rads). These cells are pulsed with antigen (e.g., 0.1-1.0 μ M) for 2 hours, and then T cells are added in 12 replicates at 1×10^5 /cells per well. Con-A (5 μ g/ml) is used as a positive control. After 4 days 1 μ Ci of 3 H-Thymidine is added to each well, and the cells are harvested 18-24 hours later. If the cells are anergic they will not proliferate in response to antigen stimulation. Alternatively, the production of IL-2 can be measured in the supernatants of the cultures described above. Supernatants are collected daily and IL-2 production is measured using a commercial ELISA assay. An additional approach includes flow cytometry based staining specific for intracellular expression of the cytokines IL-2, γ IFN and TNF α using antibodies specific to the human forms of these factors (Becton Dickinson). Further, semiquantitative RT-PCR of mRNA for these factors can also be used.

According to another aspect of the invention, a method for identifying an agent suspected of affecting hematopoietic cell development is provided. The method involves introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix of the invention, and co-culturing in a test co-culture the hematopoietic progenitor cells and the lymphoreticular stromal cells in the presence of at least one candidate agent suspected of affecting hematopoietic cell development. By "hematopoietic cell development" it is meant to include hematopoietic progenitor cell maintenance, expansion, differentiation, and/or cell-death apoptosis (programmed cell-death). "Maintenance" includes the hematopoietic progenitor cell's ability to maintain its pluripotentiality. "Expansion" includes the hematopoietic progenitor cell's ability to divide and grow, and "differentiation" includes the hematopoietic progenitor cell's ability to differentiate toward a specific cell lineage. "Cell-death" also includes programmed cell-death (apoptosis). By "affecting" hematopoietic cell development it is therefore meant to include effects on hematopoietic progenitor cell maintenance, expansion, differentiation, and/or cell-death. Such effect (or influence) can be either positive or negative/inhibitory in nature. For example, a positive effect would be maintenance of pluripotentiality of the progenitor cells, and/or increase in the number of the pluripotential progenitor cells. A negative effect would lead into the differentiation of the progenitor cells and loss of pluripotentiality, or even progenitor cell-death. A negative effect on a particular cell population may also have a positive effect on a different cell population. For example, an inhibitory effect on a B cell lineage may result in a positive effect on, for example, a T cell lineage. The agent suspected of affecting hematopoietic cell development may be administered in the form of a transfected

nucleic acid into the lymphoreticular stromal cells as well as being added straight into the media.

To determine whether the at least one candidate agent affects hematopoietic cell development in the test co-culture, the phenotype and/or genotype (as well as the numbers) of the hematopoietic cells generated in the test co-culture is compared to the phenotype and/or genotype (and numbers) of hematopoietic cells generated in a control co-culture. The control co-culture is performed under identical conditions to the test co-culture (i.e., identical initial numbers and types of both hematopoietic progenitor cells and lymphoreticular stromal cells, in an identical matrix, identical culture media, etc.), but with the exception that the at least one candidate agent suspected of affecting cell hematopoietic cell development is omitted from the control co-culture. Methods for determining the phenotype and/or genotype of hematopoietic cells are well known in the art, and a few examples can be found throughout this application.

In yet another aspect of the invention, a method for isolating from a cell culture an agent suspected of affecting hematopoietic cell development is also provided. The method involves introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix of the invention, co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells and obtaining a test-supernatant (or a fraction thereof) from the co-culture. The test-supernatant (or a fraction thereof) is then compared to a control-supernatant (or a fraction thereof). By "comparing" it is meant that a profile of agents (suspected of affecting hematopoietic cell development) present in the test-supernatant and secreted from the cells of the co-culture, is compared to a similar profile of agents present in the control-supernatant and secreted from the cells of a control culture or co-culture. Methods of obtaining such profiles of secreted agents are well known in the art and include two-dimensional (2-D) gel electrophoresis. Other methods also include various types of HPLC, thin layer chromatography.

A "control culture or co-culture" may involve the culture of hematopoietic progenitor cells

in a parallel culture system known in the art (e.g. U.S. Patent No. 5,677,139 by Johnson *et al.*), in order to obtain a result that correlates (i.e. approximates) to the result established in the co-culture system of the invention. For example, a test co-culture according to the invention that involves the co-culture of human hematopoietic progenitor cells and lymphoreticular stromal cells from a mouse thymus, gives rise to a diverse (a variety of sub-types) population

of human lymphoid cells committed to the T cell lineage. The test-supernatant obtained from such co-culture is then compared to a control-supernatant obtained from a culture of human hematopoietic progenitor cells in a parallel system of the prior art (as described above) that also gives rise to a population of human lymphoid cells committed to the T cell lineage.

5 Other examples of control cultures or co-cultures may include the co-culture of hematopoietic progenitor cells with lymphoreticular stromal cells of different tissue origin to the ones used in the test co-culture in the matrix of the invention. Additionally, the tissue may or may not be of lymphoid origin. A person of ordinary skill in the art would be able to easily choose and establish such control cultures or co-cultures. Once the profiles of agents suspected of
10 affecting hematopoietic cell development are obtained, a subfraction of the test-supernatant that contains an agent suspected of affecting hematopoietic cell development that appears to be different or absent from the control-supernatant, can then be isolated and further characterized. For example, a candidate agent that appears to be migrating differently in a 2-D gel electrophoresis blot of the test-supernatant can be purified and further characterized
15 using methods such as protein sequencing and mass spectrometry. Agents that appear in the 2-D gel electrophoresis blot but are absent from the blot of the test-supernatant are also suspect of affecting hematopoietic cell development and can be further purified.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention
20 and are not to be construed to limit the scope of the invention.

Examples

Experimental Procedures

Isolation of Human CD34⁺ cells

Five to ten milliliters of venous umbilical cord blood (UCB) was extracted using a
25 heparinized syringe prior to the severing of the umbilical cord during a Caesarean section delivery of a human embryo. After the umbilical cord was severed and the infant delivered, the placenta was removed by clamping the umbilical vein proximally and severing distally to the placenta. Immediately after the placenta was removed the umbilical vein was unclamped and the blood contained in the placenta drained into an appropriate heparinized container.
30 Before processing, the cord and placenta blood was mixed together. After extraction the cord/placenta blood was diluted 2:1 with washing media (RPMI 1640, 10 IU/ml penicillin, 10 µg/ml streptomycin, 1 mM L-glutamine). The sample(s) were then underlayered with a volume of Ficoll-Hypaque (1.077 g/ml) equal to half of the diluted sample volume so that a distinct

sample/Ficoll interface formed. After centrifugation for 45 minutes at 400 x g the interface containing mononuclear cells was removed. The cells were then washed by resuspending in culture medium and centrifuging for 10 minutes at 400 x g. The resulting pellet was resuspended in 6 ml of ammonium chloride lysing buffer (0.15M NH₄Cl, 1.0 mM KHCO₃,
5 0.1M Na₂EDTA) for 3 minutes to lyse any remaining erythrocytes. The suspension was then diluted with media and washed twice more. After the final wash the cells were resuspended in 1-2 ml media and the number of viable cells was determined by trypan blue exclusion.

Human CD34⁺ progenitor cells were also prepared from disaggregated human fetal thymus obtained from 16-22 week old abortuses. For dissagregation procedures see below
10 under *Mouse Thymic Stroma*.

Cells expressing the surface antigen CD34 were isolated using the Dynal CD34 Progenitor Cell Selection System (Dynal, Lake Success, N.Y.) or the MiniMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). The mononuclear cells isolated from UCB (or bone marrow) were suspended in isolation buffer (PBS, 2% heat inactivated fetal bovine
15 serum, 10 IU/ml penicillin, 10 µg/ml streptomycin) at a concentration of 2.5×10^7 cells/ml. The suspension was then added to magnetic anti-human CD34 beads (Dynal M-450 CD34) in a ratio of 4.0×10^7 beads per ml of suspension, in a round bottom tube. (Dynabeads M-450 CD34 are superparamagnetic beads bound to monoclonal antibody specific for CD34). The mixture was vortexed gently and incubated at 4° C for 45 minutes with gentle tilt rotation
20 using a Dynal Sample Mixer. After incubation the bead/cell mixture was resuspended in a larger volume of isolation buffer and placed in a magnetic separation device for 2 minutes to allow the cell/bead complexes to accumulate to the tube wall. While still exposed to the agent, the suspension containing the cells not bound to the magnetic beads was aspirated. The cell/bead complexes were washed three more times in this manner, pooling the suspensions
25 containing the CD34 negative cells into the same tube. The tube containing the released cells (CD34-) was then placed on the magnetic separator to remove any remaining beads and this supernatant was transferred to a new conical tube. All CD34⁺ cells attached to beads were washed twice in a minimum of 10 ml of isolation buffer with centrifugation at 2000 rpm for 8 min. Cells bound to magnetic beads were then resuspended in 100 µl of isolation buffer per
30 4×10^7 beads used, with a minimum volume of 100 µl. The CD34 positive cells were then detached from the beads by adding an equal volume of an anti-idiotypic antibody (DETACHaBEAD CD34, Dynal), vortexing, and gently mixing at room temperature using a

Dynal Sample Mixer for one hour. The cells were isolated from the cell/bead suspension by adding isolation buffer and placing the tube in the magnetic separation device for 2 minutes. After the beads migrated to the tube wall, the supernatant containing the CD34 positive cells was transferred to a new tube. The beads were washed three more times with the suspensions
5 containing the released cells pooled into the same tube. The tube containing the released CD34+ cells was then placed on the magnetic separator to remove any remaining beads, and the supernatant was transferred to a new conical tube. The cells were washed twice in a minimum of 10 ml of isolation buffer with centrifugation at 2000 rpm for 10 minutes.

Alternatively, human bone marrow was obtained by posterior iliac crest aspiration
10 from healthy adult volunteers in accordance with institutional review board guidelines and after giving informed consent. 10-15 mL of human bone marrow was collected in an heparinized sterile syringe, transported at room temperature and used within 6 hours. Bone marrow was diluted in a 5-times volume of PBS and the mononuclear cells (MNCs) separated by density gradient centrifugation over a column of Ficoll-Paque (Pharmacia Biotech Inc.,
15 Piscataway, NJ). MNCs thus obtained were washed twice in 10 mL PBS, and the remaining erythrocytes removed by lysis with ACK Lysing Buffer (Bio Whittaker, Walkersville, MD).

In order to select a more immature phenotype of progenitor cell within the CD34⁺ population, we elected to use an immunomagnetic bead selection system employing an antibody to the novel stem cell antigen, AC133. AC133 is a 5-transmembrane cell surface
20 antigen expressed on 20-60% of human CD34⁺ cells, including the CD38^{neg/dim} subset (representing the non-lineage-committed precursors) but is not expressed on mature leukocytes (Yin AH, et al., *Blood*, 1997, 90:5002-12; Nfiraglia S, et al., *Blood*, 1997, 90: 5013-21; Buhning HJ, et al., *Ann N Y Acad Sci*, 1999, 872: 25, discussion 38-9). Although a small number of mature CD2⁺ T-cells were transferred into our co-cultures with the AC133⁺
25 progenitors we do not believe that the T-cells generated in this system are derived from either CD2⁺ mature lymphocytes or CD2⁺ lymphoid-committed precursors. We, and others (Fisher AG, et al., *Int Immunol*, 1990, 2:571-8), have observed that the deliberate introduction of mature human T-cells into the co-cultures does not result in increased numbers of T-cells or their precursors. The AC133⁺ MNC fraction was isolated by immunomagnetic bead selection
30 using an AC133 Cell Isolation Kit (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer's protocols.

Mouse Thymic Stroma:

Thymi were obtained from freshly sacrificed 6 week-old B6 (BALB/C) mice. Thymi were physically disaggregated with surgical scissors in order to produce a cell suspension which also contained fragments of thymic tissue less than 0.5mm^3 in size. The cell suspension containing thymic fragments was plated onto $0.5\text{cm} \times 0.5\text{cm} \times 0.2\text{cm}$ pieces of Cellfoam (80 ppi), placed in each well of a 24 well plate. Each well contained at least 5×10^6 cells and 4 fragments of fetal thymus per Cellfoam block and the cells were cultured in fully supplemented IMDM. The medium in thymic cultures was changed initially at 48 hours post establishment of the culture and at three day intervals there after. On average 80% confluent thymic stromal monolayers were established on Cellfoam between 10 and 14 days. At 10 to 14 days of culture the Cellfoam blocks each containing a sub-confluent layer of thymic stroma were removed from the 24 well plate and placed in the wells of a new 24 well plate, and co-cultured with CD34^+ cells.

Human CD34^+ /Murine Thymic Stroma Co-Culture conditions:

Five thousand CD34^+ cells derived from UCB or human bone marrow were then plated onto the irradiated murine thymic stroma. In the case where Cellfoam was used, CD34^+ cells were plated directly onto the Cellfoam itself in the well of the 24 well tissue culture dish. Medium in co-cultures was changed every three days and was not supplemented with exogenous cytokines. Cells generated from the CD34^+ cells were harvested at 7 days post establishment of the co-culture and flow-cytometric and functional studies were performed on the derived cells.

Assessment of Immunophenotype and Function of Cells Derived from the Co-cultures:

Adherent cells were harvested with a non-trypsin isolation solution (Cell Dissociation Solution, Sigma, St. Louis, MO) to minimize alteration of surface staining characteristics. To recover adherent cells from Cellfoam, units were washed twice by immersion into PBS, saturated by brief vortexing in an excess of Cell Dissociation Solution, incubated for 20 minutes at 37°C , and centrifuged at 1500 rpm for 10 minutes.

Cells were harvested by gentle aspiration and washed twice in PBS. Harvested cells were counted and assessed for viability by trypan blue exclusion. After counting, cells were stained in a final volume of $100\mu\text{L}$ with 2% mouse serum (Dako, Carpinteria, CA) and the following fluorochrome-conjugated antibodies: $\text{TCR}\alpha\beta$, $\text{TCR}\gamma\delta$, CD2, CD3, CD4, CD8, CD14, CD33 and CD34 (Becton Dickinson, San Jose, CA). Conjugated isotype control antibodies for all four fluorochromes (FITC, PE, Peridinin chlorophyll protein (PerCP), and

Allophycocyanin (APC) were used for each culture. Stained samples were washed three times with PBS, fixed with 1% paraformaldehyde, and analyzed with a FACScalibur flow cytometer (Becton Dickinson). Appropriate controls included matched isotype antibodies to establish positive and negative quadrants, as well as appropriate single color stains to establish compensation. For each sample, at least 10,000 list mode events were collected. Anti-CD3 and anti-CD14 were utilized to detect contaminating T-cells and monocytes in the CD34⁺ selected MC subpopulation.

Human leukocytes were distinguishable from murine cells on immunophenotypic analysis by gating on the CD45⁺ population. After 14 days in co-culture, >70% of CD45⁺ cells coexpressed CD3, CD4, and/or CD8. It was possible to track the sequential differentiation of T-lymphoid precursors in this system over 2 weeks (Figure 1). CD34⁺ progenitors added into co-culture with a murine thymic stroma cells, on a three-dimensional matrix (Cellfoam). Non-adherent cells were harvested 7, 14 and 21 days after establishment of the co-cultures and their immunophenotype determined by FACS analysis. The data in panel (a) demonstrate the acquisition of CD2 and the down-regulation of the hematopoietic progenitor cell marker, CD34. Acquisition of cell surface CD4 and CD8 markers occurred after 14 days in coculture; (b): discrete populations of SP CD4⁺ and SP CD8⁺ are demonstrated including their DP CD4⁺CD8⁺ precursors. Acquisition of CD4 at day 14 was associated with acquisition of CD3; (c and d): all CD4⁺ cells co-expressed CD3. CD3 was co-expressed with the majority of CD8⁺ cells; those cells which were CD3⁻CD8⁺ were found to express TCR $\gamma\delta$. TCR $\alpha\beta$ was expressed by 78% of CD3⁺ cells although a smaller population (20%) of CD3⁺ cells expressing TCR $\gamma\delta$ was also detectable (6% CD3⁺CD8⁺ TCR $\gamma\delta$, 14% CD3⁺CD8⁺ TCR $\gamma\delta$).

T cell Function:

T cell function was assessed by determining CD69 expression in response to mitogens and ³H-Thymidine uptake in response to the mitogen Con-A. T cells generated in the co-culture system were also examined for their infectability by HIV-1 and their transducability by the MFG murine retroviral vector. T cells generated from the co-culture showed expected high levels of ³H-Thymidine uptake (10 x control unresponsive cells) in response to the mitogen ConA and a four-fold increase in the expression of the activation marker CD69 as determined by flow cytometry.

HIV-1 Challenge of T-cells generated from HPC/thymic stromal co-cultures:

T-cells generated from HPCs were challenged with T-cell tropic isolate HIV_{IIIB} at a multiplicity of infection of 1. Titered stocks of HIV-1 were generated by standard means well known in the art. Samples of culture supernatant were removed from cultures at 3, 6, 9, 14 and 28 days post HIV challenge for HIV-1 p24 antigen estimation by ELISA (Coulter, Miami, FL). Secondly, sorted CD4⁺ T cells generated from co-cultures of BM HPCs with thymic stroma were challenged with HIV_{IIIB} at a multiplicity of infection of 1. Cell viability was also determined following challenge of monocytes and T cells with HIV-1 using trypan blue exclusion. T cells generated from HPCs were infectable with HIV-1 and produced up to 0.69ng/ml of HIV-1 p24 by day 10 of culture. Both unsorted and sorted T cells generated from the co-cultures of HPCs on murine thymic stroma on Cellfoam and exposed to heat inactivated HIV_{IIIB}, produced undetectable levels of HIV-1 p24. The viability of T cells also declined significantly following exposure to infections HIV-1. The levels of HIV-1 p24 antigen production in T cells generated from the Cellfoam co-culture system was similar to levels of HIV-1 p24 production from human activated peripheral blood T cells.

Transduction of T cells generated from HPC/thymic stromal co-cultures with an amphotropic murine retroviral vector:

T cells generated from HPCs and expanded in IL-2 and PHA were exposed to the murine retrovirus based vector, MFG, encoding the intranuclear localized enzyme β -galactosidase at an M.O.I. of 10 on three occasions over the period of 72 hours. Titered retroviral vector was generated by standard means from a human based FLYA4 packaging cell-line. T cells were also exposed to heat inactivated MFG. Transduced cells were harvested from cultures at 7 days following retroviral exposure and stained by standard methods for the expression of the beta-galactosidase transgene. Transduction efficiencies of between 12 and 26% were observed in T cells generated from co-cultures of HPCs with murine thymic stroma grown on Cellfoam. No β -galactosidase activity was detectable in T cells exposed to the heat inactivated retroviral vector. The transduction efficiency of human T cells generated from the Cellfoam co-culture system is similar to that seen in activated peripheral blood T cells.

mRNA extraction and cDNA synthesis:

Generated cells were also lysed and RNA was prepared from the cells for RNA PCR in order to determine T cell receptor gene expression. Messenger RNA was extracted from cells grown on a thymic monolayer. The extraction was performed using guanidinium thiocyanate and oligo-dT spun columns (QuickPrep Micro mRNA Purification Kit;

Pharmacia, Piscataway, N.J.) according to the manufacturer's instructions. mRNA samples were stored at -70° C. The first strand cDNA was synthesized in a 40 µl final volume, using approximately 2 µg of mRNA, 1 µg of random primer, and 6.25 units of AMV reverse transcriptase (GIBCO/BRL). Samples were incubated for 10 minutes at room temperature, 1 hour at 42° C., 5 minutes at 95° C., and 5 minutes at 4° C. RT-PCR for a number of lymphoid-specific genes (including RAG-2) was performed using reverse transcription using random primers and Moloney MuLV reverse transcriptase (GIBCO-BRL, Grand Island, NY). cDNAs were amplified using gene-specific primers, e.g., for the human RAG-2 gene which is expressed transiently only by cells undergoing lymphocyte differentiation, Vβ gene expression, and the like. PCR amplification were performed in a GeneAmp 9600 thermal cyclor (Perkin Elmer, Norwalk, CT) using conditions well known in the art.

Example 1: *Viability, Immunophenotype and Function of Human Cells Generated In Co-Culture Systems*

The numbers of viable cells generated in the co-culture system and their immunophenotype are shown in Table 2. Maximal human T cell proliferation was seen when human fetal thymic CD34⁺ cells and UCB CD34⁺ cells were co-cultured with murine fetal thymic stroma grown on Cellfoam. Data generated from a direct comparison of co-culture of CD34⁺ cells on murine thymic stroma on cell foam versus co-culture of CD34⁺ cells on murine stroma grown as a simple monolayer are also shown in Table 2.

T cells generated in the co-culture system were also shown to be infectable by T-tropic HIV-1_{IIIB} and these cells were also transductible at a transduction efficiency of 12-22% (n=3) with MFG vector.

Example 2: *Maintenance of Immature Progenitor Cells*

According to the invention, it has also been discovered that Cellfoam cultures of thymic stromal cells are able to induce T cell differentiation of CD34⁺ progenitors and yet preserve a fraction of CD34⁺ cells. Primate CD34⁺ progenitors were cultured on either human or swine thymus that had been established on Cellfoam tissue scaffolds. After 14-21 days, CD3⁺CD4⁺CD8⁺ triple positive cells and CD3⁺CD4⁺ and CD3⁺CD8⁺ double positive cells are reliably recovered. In addition, the CD3⁻ cell fraction was found to contain CD34⁺ progenitor cells after 14-21 days. These CD34⁺ cells not only were CD3⁻, but many were also CD2⁺. This demonstrates that thymus cultures in Cellfoam tissue scaffolds can support T cell differentiation while simultaneously preserving the long-lived CD34⁺ progenitor cell population. As will be evident to those skilled in the art, this surprising finding indicates that

ongoing differentiation of T progeny while maintaining immature progenitor cells is possible in Cellfoam.

Example 3: T Cell Function (Proliferation/Anergy) Assays

T cell function is evaluated by the proliferative potential to specific and non-specific antigens using standard assays. Specifically, the assay assesses the response of T cell receptor (TCR) mediated proliferation using anti-CD3 antibodies (Becton Dickinson) as well as baseline non-specific proliferation using concavalin A (Con-A). Briefly, T cells are washed and resuspended in RPMI with 10% FCS at a concentration of 10^6 cells/ml. 100 μ l (10^5 cells) are added to each well of a 96 well plate. Cells are stimulated with either Con-A (5 μ g/ml) (non-specific response) or monoclonal antibodies to CD3 in the presence of IL-2 (20 units/ml) and irradiated mononuclear cells (MCs) (10^5 cells/well in 100 ml of RPMI with 10% FCS). Purified goat anti-mouse F(ab')₂ fragments (Kirkegard and Perry Laboratories, Gaithersburg, MD) are used as a crosslinking agent for the experimental conditions where monoclonal antibodies to CD3 are used. Wells are pretreated with 1.25 μ g/ml of goat anti-mouse antibody for 45 minutes at 37°C and washed three times prior to the addition of monoclonal antibodies to CD3 and CD28. Controls included T cells alone, T cells plus irradiated MCs, and T cells plus mitogenic stimuli without IL-2 or irradiated MC. After 7 days in culture at 37°C, cell proliferation are assessed using either radio-active assays or commercially available non-radioactive, ELISA based assays (e.g. Promega). Cells are co-cultured for 5-7 days to induce proliferation of the T cells (the stimulator cells are also irradiated and thus non-proliferative). Stimulator cells alone serve as controls.

An additional approach to testing T cell function uses flow cytometry based staining for intracellular expression of the cytokines IL-2 γ IFN and TNF α using antibodies specific to the human forms of these factors (Becton Dickinson). These cytokines are produced in the T progeny in the antigen specific *in vitro* proliferation assays. This allows low level detection of human cells among a high proportion of mouse cells, selectively highlighting the human progeny and excluding the mouse cells. Further, semiquantitative RT-PCR of mRNA for these factors can also be used.

In one particular example, for instance, cells removed from co-culture after 14 days showed pronounced proliferation when placed in liquid culture with complete medium and IL-2 (10 IU/mL) and phytohemagglutinin (PHA; 2 μ g/mL). After a further 7 days in culture there was a 45-fold increase in cell number: >90% were CD3⁺CD4⁺ TCR $\alpha\beta$ ⁺; 3% CD3⁺CD8⁺ TCR $\alpha\beta$ ⁺ and 3% CD3⁺CD8⁺CD4⁺ TCR $\alpha\beta$ ⁺. No cells expressing TCR $\gamma\delta$ were detected.

TABLE 2. - USE OF THYMIC STROMA/HUMAN FETAL CD34+CELL CO-CULTURE SYSTEM

	Standard Protocol n=3 Human UCB CD34+ Human fetal thymic stroma		Fetal Human Thymic stroma	6 Week Murine Thymic stroma	Neonatal swine Thymic stroma	Fetal Human Thymic stroma	6 Week Murine Thymic stroma	Neonatal Swine Thymic stroma
			n=3 Cell foam Thy CD34+	n=3 Cell foam Thy CD34+	n=3 Cell foam Thy CD34+	n=3 Monolayer Thy CD34+	n=3 Monolayer Thy CD34+	n=3 Monolayer Thy CD34+
	d14	d28	d7	d7	d7	d7	d7	d7
CD4	2.8	3.8	81.54	85.8	67.8	88.2	81.1	69.2
CD3	1.5	1.1	12.75	87.1	80.7	13.1	90.1	79.2
CD4/CD3	4.2	1.4	9.83	75.2	64.65	10.6	68.1	66.3
CD8/CD3	2.1	2.8	0.13	74.5	28.8	0.46	71.9	28.3
CD4/CD8	1.8	1	0.37	79.5	25.4	0.89	89.1	22.9
CD2	4.1	12.5	ND	23.2	50.7	ND	20.4	48.1
CD14	33.4	16.1	59.6	0.17	6.8	63.1	0.81	4.6
CD33	48.2	20.2	90.61	0.33	ND	84.1	0.59	ND
CD2/CD14	1.6	7.3	ND	0.85	ND	ND	0.26	ND
CD2/CD33	4.8	12.8	ND	3.76	ND	ND	4.12	ND
CD33/CD14		8.4	60.73	0.13	ND	56.13	0.11	ND
Viable Cell Count								
t ₀ =5,000	195,000	210,000	98,000	1,800,000	220,000	15,000	79,000	51,000

Example 4: T Cell Lymphopoiesis Assay

AC133⁺ progenitor cells were added to the murine thymic stromal cultures at cell densities of either 1×10^5 , 1×10^4 , or 1×10^3 cells per well and cultured for an additional two weeks at 37°C in a 5% CO₂ humidified atmosphere. Medium in the co-cultures was changed every 4 days and was not supplemented with exogenous cytokines. Cells generated from the precursors were harvested 7 and 14 days after establishment of the co-cultures.

The selected AC133⁺ cells represented a highly purified progenitor cell population. Immunophenotypic analysis showed that >98% were CD34⁺; none co-expressed surface CD3, CD4 or CD8. A small number of contaminating CD2⁺ cells were detectable by flow cytometry within the AC133⁺ selected population: this comprised only $0.57\% \pm 0.29\%$ (mean \pm SEM; n=6) of the cells obtained from the selection process.

Example 5: Determination of Optimum Matrix Size and Input Cell Number

Having tested matrices of differing dimensions we have determined that the optimal sized matrix for use in this system measures 10mm diameter x 1 mm in depth. Similarly, input cell density appears critical for optimum T-cell generation: no lymphocytes were generated using an input cell density of less than 1×10^4 cells per well. However, using 10 x 1 mm matrices and input cell densities of 1×10^4 or 1×10^5 , we were able to generate large numbers of human cells, $71.21\% \pm 9.87\%$ (mean SEM; n=7) of which were CD3⁺, after 14 days in co-culture.

Example 6: Intra- and Inter-Sample Variability in Numbers of T-Cells Generated

In order to determine the variation of T-cell output within the a given source of progenitors, multiple co-cultures were established using a single source of AC133⁺ cells at fixed cell densities (1×10^4 cells per well) on separate 10 x 1 mm matrices of murine thymic stroma. The intrasample variation of T lymphocytes generated was analyzed by cell count using trypan blue exclusion, and by immunophenotypic analysis. Human cells were distinguished by surface expression of CD45. After 7 days in co-culture, the number of mature T-cells detected was extremely low: CD3⁺ cells represented $2.02\% \pm 0.87\%$ (mean \pm standard error) of the CD45⁺-gated population, CD3⁺CD4⁺ T-cells accounted for $1.0\% \pm 0.52\%$ of the gated population and CD3⁺CD8⁺ $0.58\% \pm 0.1\%$ of the same gated population. However, after 14 days, the numbers of T-cells were significantly higher: the proportion of

CD3⁺ cells rose to 62.16% ± 4.53%; and the percentages of CD3⁺CD4⁺ and CD3⁺CD8⁺ were 42.7% ± 2.87% and 22.39% ± 1.29% respectively. These data are represented graphically in figure 2.

The intersample variation was calculated by comparing the number of T-cells generated from separate sources of CD34⁺ progenitors. In each case a fixed number of cells (1 x 10⁴ cells per well) had been introduced into co-culture. Immunophenotypic analysis of cells generated after 7 days in co-culture showed that, of the CD45 gated population, 1.57% ± 0.97% of cells expressed CD3; 2.27% ± 2.70% co-expressed CD3 and CD4; and 0.46% ± 0.23% expressed both CD3 and CD8. After 14 days, the immunophenotype of the cells harvested revealed that 71.21% ± 9.87% were CD3⁺; 37.44% ± 8.44% were CD3⁺CD4⁺, and 38.06% ± 19.13% were CD3⁺CD8⁺ as shown in figure 3.

These data demonstrate a high level of reproducibility within the system that suggests its potential in comparative analyses of input populations.

Example 7: Analysis for TCR Excision Circles (TREC)

The TCR *Vδ* locus lies between the TCR *Vα* and TCR *Jα* segments. In order to complete TCRα *VD-J* rearrangement, the TCR *Vδ* segment is excised: the 3' and 5' ends of the gene unite to form an extra-chromosomal circle of DNA termed a TCR excision circle (TREC) (Berenson RJ, *et al.*, *J Clin Invest*, 1988, 81: 951-5; Broxmeyer HE, *et al.*, *Proc Natl Acad Sci USA*, 1989, 86:3828-32). TRECs do not duplicate when the T-cell divides (Blom B., *et al.*, *J Immunol*, 1997, 158:3571-7). As a consequence, TREC levels are highest in recent thymic emigrants but are sequentially diluted amongst the emigrants' progeny. TCRδ TRECs are detectable by PCR - an assay that has been shown to be a reliable tool for monitoring *de novo* T-cell generation (Tjormford GE, *et al.*, *J Exp Med*, 1993, 177:1531-9). Absolute numbers of TREC positive cells will vary according to the total number of cells analyzed. We determined that the significance of TREC positivity would be most fairly interpreted by calculating the ratio of the number of TREC copies detected to the number of β-actin copies detected. We compared the level of TREC detected in T-cells harvested from the co-cultures after 14 days to TREC levels in peripheral blood mononuclear cells, B cells, AC133⁺ cells from the input population, and human fetal thymocytes. The highest TREC:bactin ratio was found in T-cells generated from the co-cultures after 14 days (0.54), followed by thymocytes from 16-22 week human fetuses (0.017). The TREC:bactin ratios from fetal and adult PBMCs

and from AC133⁺ bone marrow mononuclear cells was significantly lower. These data are summarized in Table 3, below. No TREC was detected in any of the samples of B-cells tested (n=6).

Table 3.

Source	n	TREC/Bactin ratio (mean)
Murine Thymocytes	6	0
Bone Marrow AC133+ progenitors	3	0.000014
Adult Peripheral blood MNCs	3	0.00141
Fetal Peripheral blood MNCs	1	0.0024
Fetal Thymocytes	2	0.017
T-Cells generated <i>in vitro</i>	2	0.54

These data conclusively demonstrate that rearrangement of TCR occurs during the course of the culture period. The abundance of TREC positive cells compares favorably with that seen from fresh fetal thymus and supports the physiologic equivalence of the *in vitro* system in this aspect of T-cell differentiation.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references disclosed herein are incorporated by reference in their entirety.

We claim:

Claims

1. A method for *in vitro* production of lymphoid tissue-specific cells, comprising:

introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix, wherein the amount of the lymphoreticular stromal cells is sufficient to support the growth and differentiation of the hematopoietic progenitor cells, and

co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells.

2. The method of claim 1, wherein the co-culturing occurs under conditions sufficient to produce at least a 10-fold increase in the number of lymphoid tissue origin cells.

3. The method of claim 1, wherein the co-culturing occurs under conditions sufficient to produce at least a 20-fold increase in the number of lymphoid tissue origin cells.

4. The method of claim 1, wherein the co-culturing occurs under conditions sufficient to produce at least a 50-fold increase in the number of lymphoid tissue origin cells.

5. The method of claim 1, wherein the co-culturing occurs under conditions sufficient to produce at least a 100-fold increase in the number of lymphoid tissue origin cells.

6. The method of claim 1, wherein the co-culturing occurs under conditions sufficient to produce at least a 200-fold increase in the number of lymphoid tissue origin cells.

7. The method of claim 1, wherein the co-culturing occurs under conditions sufficient to produce at least a 400-fold increase in the number of lymphoid tissue origin cells.

8. The method of claim 1, wherein the hematopoietic progenitor cells are selected from the group consisting of pluripotent stem cells, multipotent progenitor cells and progenitor cells committed to specific hematopoietic lineages.

9. The method of claim 8, wherein the progenitor cells committed to specific hematopoietic lineages are committed to a lineage selected from the group consisting of T cell lineage, B cell lineage, dendritic cell lineage, Langerhans cell lineage and lymphoid tissue-specific macrophage cell lineage.

10. The method of claim 1, wherein the hematopoietic progenitor cells are derived from tissue selected from the group consisting of bone marrow, peripheral blood, umbilical cord

blood, placental blood, lymphoid soft tissue, fetal liver, embryonic cells and aortal-gonadal-mesonephros derived cells.

11. The method of claim 10, wherein the lymphoid soft tissue is selected from the group consisting of thymus, spleen, liver, lymph node, skin, tonsil and Peyer's patches.

5 12. The method of claim 9, wherein the lymphoreticular stromal cells are thymic stromal cells and the progenitor cells committed to specific hematopoietic lineages are committed to a T cell lineage.

13. The method of claim 1, wherein the hematopoietic progenitor cells are genetically altered hematopoietic progenitor cells.

10 14. The method of claim 1, wherein the lymphoreticular stromal cells are derived from at least one lymphoid soft tissue selected from the group consisting of thymus, spleen, liver, lymph node, skin, tonsil and Peyer's patches, and combinations thereof..

15. The method of claim 1, wherein the lymphoreticular stromal cells are genetically altered lymphoreticular stromal cells.

15 16. The method of claim 1, wherein the lymphoreticular stromal cells are seeded prior to inoculating the hematopoietic progenitor cells.

17. The method of claim 1, wherein the lymphoreticular stromal cells are seeded at the same time as the hematopoietic progenitor cells.

20 18. The method of claim 1, wherein the hematopoietic progenitor cells are of human origin and the lymphoreticular stromal cells are of human origin.

19. The method of claim 1, wherein the hematopoietic progenitor cells are of human origin and the lymphoreticular stromal cells are of nonhuman origin.

20. The method of claim 19, wherein the nonhuman origin lymphoreticular stromal cells are of murine origin.

25 21. The method of claims 1-20, wherein the porous solid matrix is an open cell porous matrix having a percent open space of at least 75%.

22. The method of claim 21, wherein the porous solid matrix has pores defined by interconnecting ligaments having a diameter at midpoint, on average, of less than 150 μ m.

30 23. The method of claim 22, wherein the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material.

24. The method of claim 23, wherein the metal is selected from the group consisting of tantalum, titanium, platinum, niobium, hafnium, tungsten, and combinations thereof, wherein

said metal is coated with a biological agent selected from the group consisting of collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitronectin, antibodies and fragments thereof, and combinations thereof.

25. The method of claim 24, wherein the porous, solid matrix having seeded
5 hematopoietic progenitor cells and their progeny, and lymphoreticular stromal cells, is impregnated with a gelatinous agent that occupies pores of the matrix.

26. The method of claim 24, wherein the metal is tantalum.

27. The method of claim 1, wherein the hematopoietic progenitor cells and the
10 lymphoreticular stromal cells are cultured in an environment that is free of stromal cell conditioned medium and exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion, and differentiation, other than serum.

28. The method of claim 27, wherein the hematopoietic growth factors that promote
15 hematopoietic cell maintenance, expansion, and differentiation, are agents selected from the group consisting of interleukin 3, interleukin 6, interleukin 11, SCF, FLT/FLK ligand growth factors.

29. The method of claim 1, wherein the hematopoietic progenitor cells and the
20 lymphoreticular stromal cells are cultured with an exogenously added agent selected from the group consisting of stromal cell conditioned medium, and a hematopoietic growth factor that promotes hematopoietic cell maintenance, expansion, differentiation, and influences cell localization.

30. The method of claim 29, wherein the hematopoietic growth factor that promotes
25 hematopoietic cell maintenance, expansion, differentiation, and influences cell localization, is an agent selected from the group consisting of interleukin 3, interleukin 6, interleukin 7, interleukin 11, interleukin 12, stem cell factor, FLK-2 ligand, FLT-2 ligand, Epo, Tpo, GM-CSF, G-CSF, Oncostatin M, and M-CSF.

31. The method of claim 1, further comprising:

after the co-culturing step, harvesting the lymphoid tissue origin cells.

32. A method for *in vivo* maintenance, expansion and/or differentiation of hematopoietic
progenitor cells, comprising:

30 implanting into a subject a porous, solid matrix having seeded hematopoietic progenitor cells and lymphoreticular stromal cells,

wherein the porous, solid matrix is an open cell porous matrix having a percent open space of at least 75%.

33. The method of claim 32, further comprising the porous, solid matrix having seeded hematopoietic progenitor cells and lymphoreticular stromal cells by the steps of:

5 introducing *in vitro* an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into the porous, solid matrix;

co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells in an environment that is free of stromal cell conditioned medium and exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion and/or differentiation, other than serum.

10 34. The method of claim 33, wherein the co-culturing occurs under conditions sufficient to produce an increase in the number of lymphoid tissue origin cells of at least between 10-fold and 400-fold.

35. The method of claim 32, wherein the hematopoietic progenitor cells are selected from the group consisting of pluripotent stem cells, multipotent progenitor cells and progenitor cells committed to specific hematopoietic lineages.

15 36. The method of claim 35, wherein the progenitor cells committed to specific hematopoietic lineages are committed to a lineage selected from the group consisting of T cell lineage, B cell lineage, dendritic cell lineage, Langerhans cell lineage and lymphoid tissue-specific macrophage cell lineage.

20 37. The method of claim 36, wherein the lymphoreticular stromal cells are thymic stromal cells and the progenitor cells committed to specific hematopoietic lineages are committed to a T cell lineage.

25 38. The method of claim 32, wherein the porous solid matrix has pores defined by interconnecting ligaments having a diameter at midpoint, on average, of less than 150 μ m.

39. The method of claim 38, wherein the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material.

30 40. The method of claim 39, wherein the metal is selected from the group consisting of tantalum, titanium, platinum, niobium, hafnium, tungsten, and combinations thereof, wherein said metal is coated with a biological agent selected from the group consisting of collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors,

glycosaminoglycans, vitrogen, antibodies and fragments thereof, and combinations thereof.

41. The method of claim 40, wherein the metal is tantalum.

42. The method of claims 32-41, wherein the porous, solid matrix having seeded
5 hematopoietic progenitor cells and lymphoreticular stromal cells is impregnated with a gelatinous agent that occupies pores of the matrix.

43. A method for inducing T cell anergy comprising:

introducing an amount of hematopoietic progenitor cells, an amount of
antigen presenting cells and an amount of lymphoreticular stromal cells into a porous, solid
10 matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix, and

co-culturing the hematopoietic progenitor cells, the antigen presenting
cells and the lymphoreticular stromal cells in the presence of at least one antigen under
conditions sufficient to induce the formation of T cells and/or T cell progenitors and to inhibit
15 activation of the formed cells.

44. The method of claim 43, wherein the hematopoietic progenitor cells are selected from
the group consisting of pluripotent stem cells, multipotent progenitor cells, and progenitor
cells committed to specific hematopoietic lineages.

45. The method of claim 44, wherein the lymphoreticular stromal cells are thymic stromal
20 cells and the progenitor cells committed to specific hematopoietic lineages are committed to a T cell lineage.

46. A method for inducing T cell reactivity comprising:

introducing an amount of hematopoietic progenitor cells, an amount of antigen
presenting cells and an amount of lymphoreticular stromal cells into a porous, solid matrix
25 having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix, and

co-culturing the hematopoietic progenitor cells, the antigen presenting cells
and the lymphoreticular stromal cells in the presence of at least one antigen under conditions
sufficient to induce from the hematopoietic progenitor cells the formation of T cells and/or T
30 cell progenitors having specificity for the at least one antigen.

47. The method of claim 46, wherein the hematopoietic progenitor cells are selected from the group consisting of pluripotent stem cells, multipotent progenitor cells, and progenitor cells committed to specific hematopoietic lineages.

48. The method of claim 47, wherein the lymphoreticular stromal cells are thymic stromal cells and the progenitor cells committed to specific hematopoietic lineages are committed to a T cell lineage.

49. The method of claim 46, wherein the antigen presenting cells are cells selected from the group consisting of dendritic cells, monocytes/macrophages, Langerhans cells, Kupfer cells, microglia, alveolar macrophages and B cells.

50. The method of claim 46, wherein the antigen presenting cells are derived from hematopoietic progenitor cells *in vitro*.

51. The method of claim 46, further comprising administering a co-stimulatory molecule to the co-culture.

52. The method of claim 51, wherein the co-stimulatory molecule is selected from the group consisting of lymphocyte function associated antigen 3 (LFA-3), CD2, CD40, CD80/B7-1, CD86/B7-2, OX-2, CD70, and CD82.

53. A composition comprising:

a porous, solid matrix having a percent open space of at least 75% and pores of a pore size sufficient to permit cells to grow throughout the matrix,

an amount of lymphoreticular stromal cells attached to the solid matrix, wherein the amount is sufficient to support the growth and differentiation of hematopoietic progenitor cells, and

an amount of hematopoietic progenitor cells attached to the matrix.

54. The composition of claim 53, wherein the hematopoietic progenitor cells are attached to the lymphoreticular stromal cells.

55. The composition of claims 53 or 54, wherein the porous solid matrix has pores defined by interconnecting ligaments having a diameter at midpoint, on average, of less than 150µm.

56. The composition of claim 55, wherein the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material.

57. The composition of claim 56, wherein the metal is selected from the group consisting of tantalum, titanium, platinum, niobium, hafnium, tungsten, and combinations thereof, wherein said metal is coated with a biological agent selected from the group consisting of

collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitrogen, antibodies and fragments thereof, and combinations thereof.

58. The composition of claim 56, wherein the metal is tantalum.

5 59. The composition of claim 53, wherein the porous solid matrix having seeded hematopoietic progenitor cells and lymphoreticular stromal cells is impregnated with a gelatinous agent that occupies pores of the matrix.

60. The composition of claim 53, wherein the hematopoietic progenitor cells are selected from the group consisting of pluripotent stem cells, multipotent progenitor cells and
10 progenitor cells committed to specific hematopoietic lineages.

61. The composition of claim 60, wherein the progenitor cells committed to specific hematopoietic lineages are committed to a lineage selected from the group consisting of T cell lineage, B cell lineage, dendritic cell lineage, Langerhans cell lineage and lymphoid tissue-specific macrophage cell lineage.

15 62. The composition of claim 60, wherein the lymphoreticular stromal cells are thymic stromal cells and the progenitor cells committed to specific hematopoietic lineages are committed to a T cell lineage.

63. A method for identifying an agent suspected of affecting hematopoietic cell development, comprising:

20 introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix,

25 co-culturing in a test co-culture the hematopoietic progenitor cells and the lymphoreticular stromal cells in the presence of at least one candidate agent suspected of affecting hematopoietic cell development, and

determining whether the at least one candidate agent affects hematopoietic cell development in the test co-culture by comparing the test co-culture hematopoietic cell development to a control co-culture whereby hematopoietic progenitor cells and
30 lymphoreticular stromal cells are co-cultured in the absence of the at least one candidate agent,

wherein the porous, solid matrix is an open cell porous matrix having a percent open space of at least 75%.

64. The method of claim 63, wherein hematopoietic progenitor cell development comprises hematopoietic progenitor cell maintenance.

5 65. The method of claim 63, wherein hematopoietic progenitor cell development comprises hematopoietic progenitor cell expansion.

66. The method of claim 63, wherein hematopoietic progenitor cell development comprises hematopoietic progenitor cell differentiation toward a specific cell lineage.

10 67. The method of claim 63, wherein hematopoietic progenitor cell development comprises hematopoietic progenitor cell-death.

68. The method of claim 63, wherein the lymphoreticular stromal cells are thymic stromal cells.

69. The method of claim 63, wherein the porous solid matrix has pores defined by interconnecting ligaments having a diameter at midpoint, on average, of less than 150 μ m.

15 70. The method of claim 63, wherein the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material.

71. The method of claim 69, wherein the metal is selected from the group consisting of tantalum, titanium, platinum, niobium, hafnium, tungsten, and combinations thereof, wherein said metal is coated with a biological agent selected from the group consisting of collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitrogen, antibodies and fragments thereof, and combinations thereof.

72. The method of claim 70, wherein the metal is tantalum.

25 73. A method for isolating from a cell culture an agent suspected of affecting hematopoietic cell development, comprising:

introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix,

30 co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells,

obtaining a test-supernatant from the co-culture,

comparing the test-supernatant to a control-supernatant, and
obtaining a subfraction of the test-supernatant that contains an agent suspected
of affecting hematopoietic cell development that is absent from the control-supernatant,

wherein the porous, solid matrix is an open cell porous matrix having a percent open
space of at least 75%.

74. The method of claim 73, wherein the agent is present in the control-supernatant and
absent from the test-supernatant.

75. The method of claim 73, wherein the agent in the test-supernatant is different from the
agent in the control-supernatant.

76. The method of claim 73, wherein the agent is further isolated by preparing a further
subfraction of the test-supernatant.

77. The method of claim 73, wherein hematopoietic progenitor cell development
comprises hematopoietic progenitor cell maintenance.

78. The method of claim 73, wherein hematopoietic progenitor cell development
comprises hematopoietic progenitor cell expansion.

79. The method of claim 73, wherein hematopoietic progenitor cell development
comprises hematopoietic progenitor cell differentiation toward a specific cell lineage.

80. The method of claim 73, wherein hematopoietic progenitor cell development
comprises hematopoietic progenitor cell-death.

81. The method of claim 73, wherein the lymphoreticular stromal cells are thymic stromal
cells.

82. The method of claim 73, wherein the porous solid matrix has pores defined by
interconnecting ligaments having a diameter at midpoint, on average, of less than 150 μ m.

83. The method of claim 73, wherein the porous solid matrix is a metal-coated reticulated
open cell foam of carbon containing material.

84. The method of claim 83, wherein the metal is selected from the group consisting of
tantalum, titanium, platinum, niobium, hafnium, tungsten, and combinations thereof, wherein
said metal is coated with a biological agent selected from the group consisting of collagens,
fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors,
glycosaminoglycans, vitrogen, antibodies and fragments thereof, and combinations thereof.

85. The method of claim 83, wherein the metal is tantalum.

Figure 1.

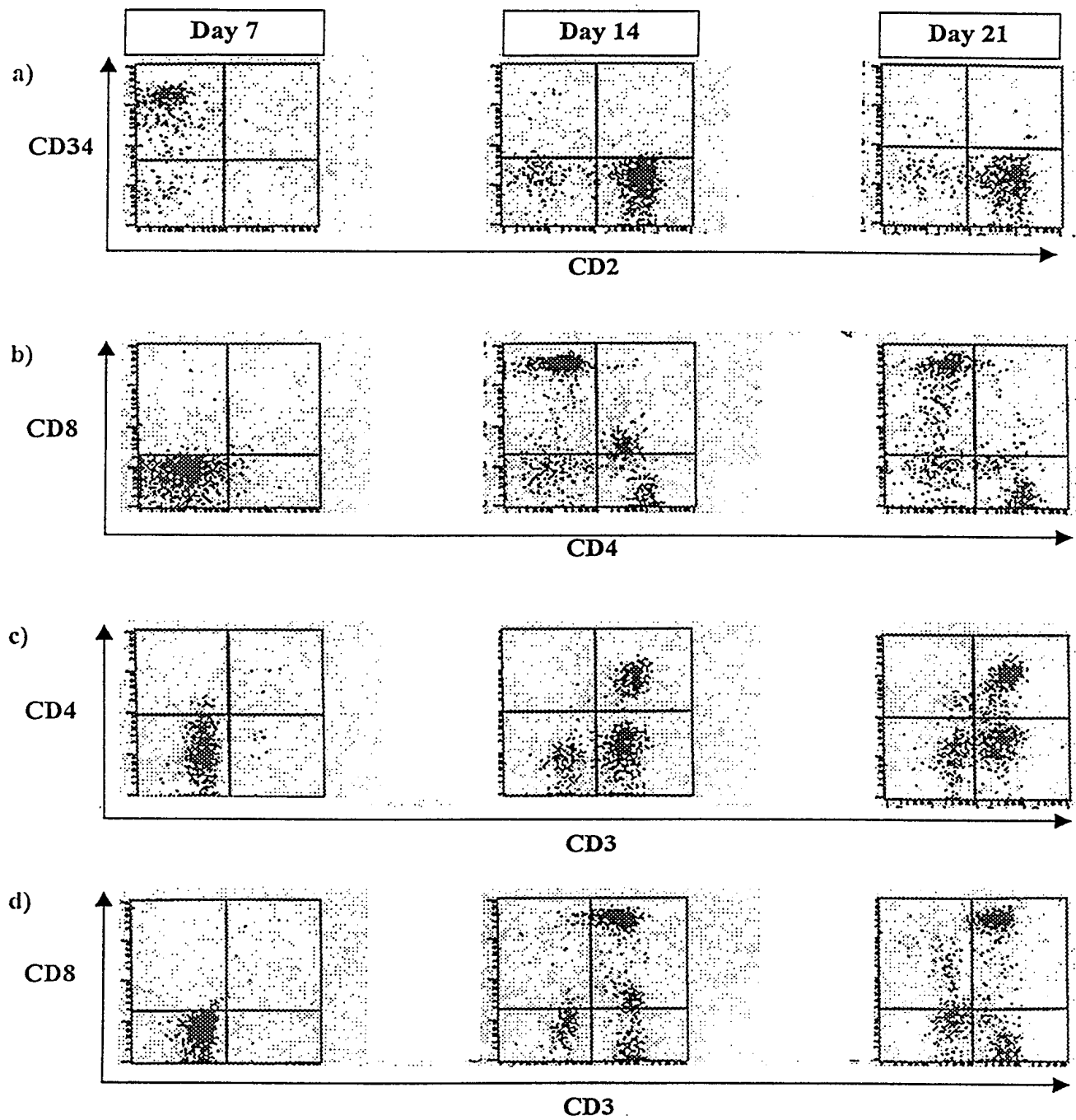


Figure 2.

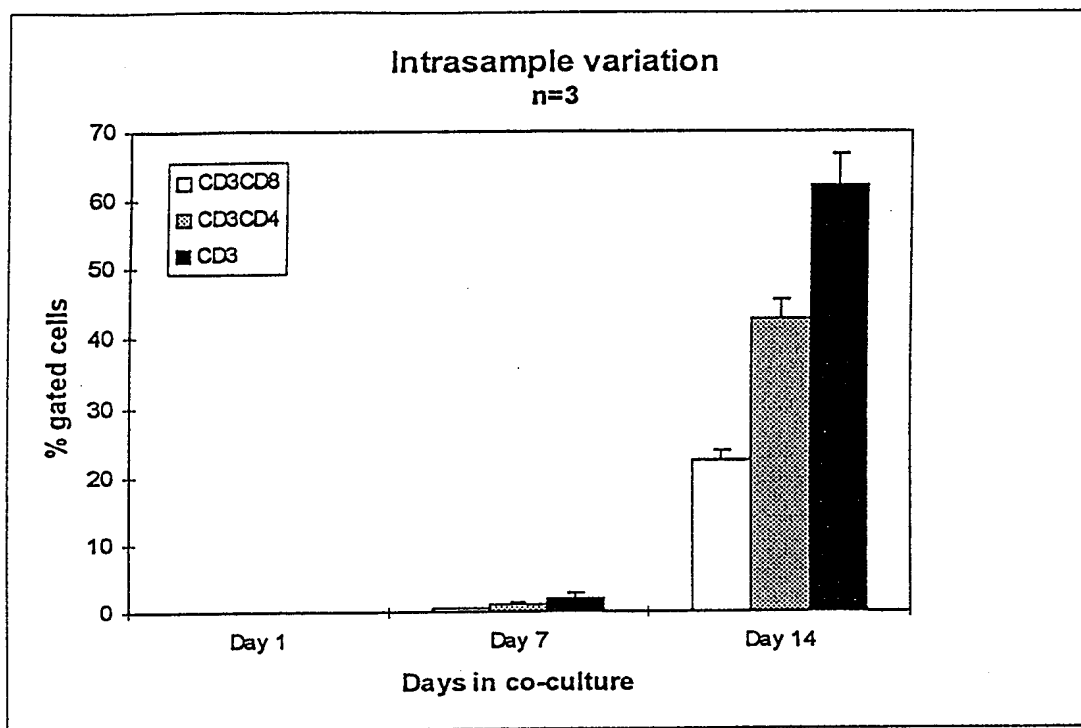
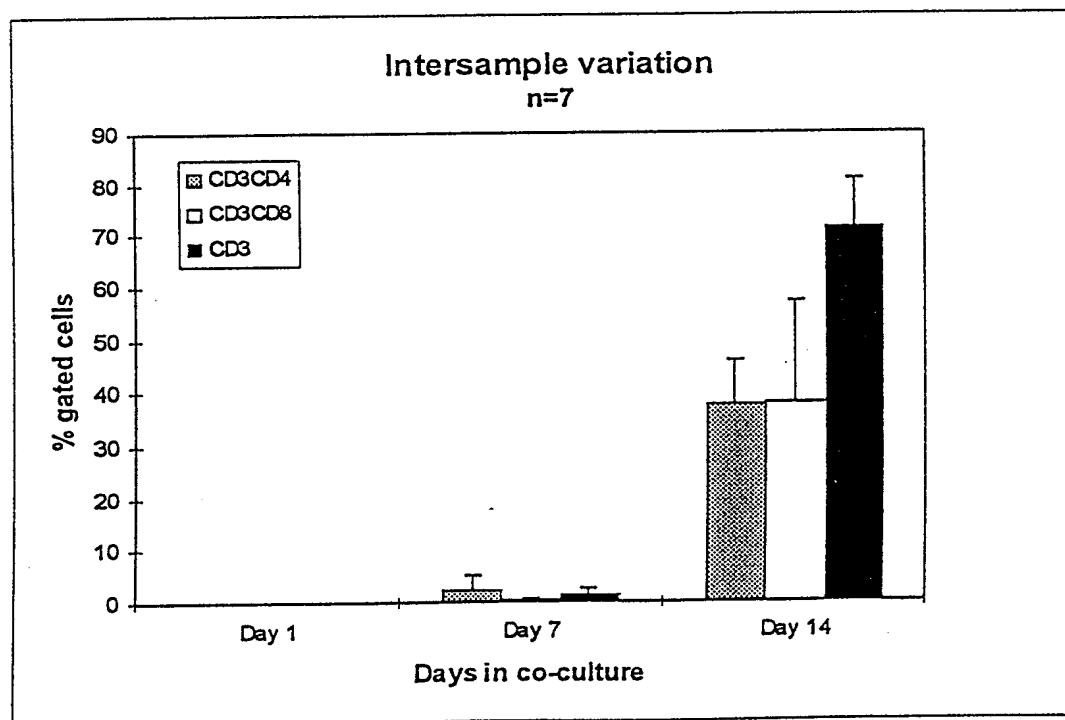


Figure 3.





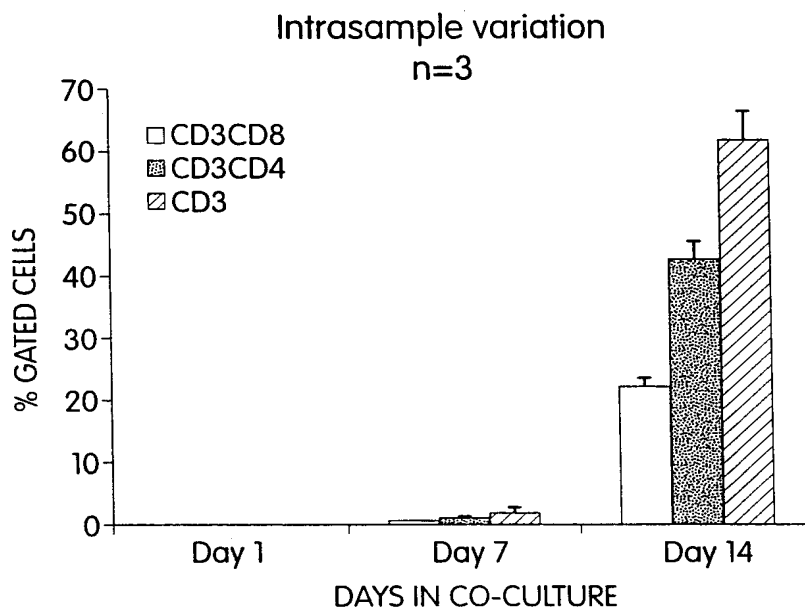
PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 5/06, 5/08, A61L 27/38		A3	(11) International Publication Number: WO 00/27999
			(43) International Publication Date: 18 May 2000 (18.05.00)
(21) International Application Number: PCT/US99/26795		(81) Designated States: AU, CA, CN, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 12 November 1999 (12.11.99)		Published With international search report.	
(30) Priority Data: 60/107,972 12 November 1998 (12.11.98) US		(88) Date of publication of the international search report: 3 August 2000 (03.08.00)	
(71) Applicants (for all designated States except US): CYTOMATRIX, LLC [US/US]; 50 Cummings Park, Woburn, MA 01801 (US). THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): ROSENZWEIG, Michael [US/US]; Cytomatrix, LLC, 50 Cummings Park, Woburn, MA 01801 (US). PYKETT, Mark, J. [US/US]; Cytomatrix, LLC, 50 Cummings Park, Woburn, MA 01801 (US). SCADDEN, David, T. [US/US]; Massachusetts General Hospital East, 13th Street, Building 149, Charlestown, MA 02129 (US). POZNANSKY, Mark, C. [US/US]; Massachusetts General Hospital East, 13th Street, Building 149, Charlestown, MA 02129 (US).			
(74) Agent: GATES, Edward, R.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).			

(54) Title: LYMPHOID TISSUE-SPECIFIC CELL PRODUCTION FROM HEMATOPOIETIC PROGENITOR CELLS IN THREE-DIMENSIONAL DEVICES



(57) Abstract

The invention relates to a method for lymphoid tissue-specific cell production from hematopoietic progenitor cells in unique, three-dimensional culture devices, in the presence of lymphoreticular stromal cells and in the absence of exogenously added growth factors. The resulting differentiated progeny. The lymphoid tissue-specific cells may be isolated at any sequential stage of differentiation and further expanded. The lymphoid tissue-specific cells also may be genetically altered at any stage of the process.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**LYMPHOID TISSUE-SPECIFIC CELL PRODUCTION FROM
HEMATOPOIETIC PROGENITOR CELLS IN THREE-DIMENSIONAL DEVICES**

Related Applications

This application claims priority under 35 U.S.C. §119 from Provisional U.S. Patent Application Serial No. 60/107,972 filed on November 12, 1998, entitled LYMPHOID TISSUE-SPECIFIC CELL PRODUCTION FROM HEMATOPOIETIC PROGENITOR CELLS IN THREE-DIMENSIONAL DEVICES. The contents of the provisional application are hereby expressly incorporated by reference.

Field of the Invention

The invention pertains to the co-culture of hematopoietic progenitor cells and lymphoreticular stromal cells in three-dimensional devices, resulting in unexpectedly high numbers of lymphoid tissue-specific cell progeny.

Background of the Invention

A characteristic of the immune system is the specific recognition of antigens. This includes the ability to discriminate between self and non-self antigens and a memory-like potential that enables a fast and specific reaction to previously encountered antigens. The vertebrate immune system reacts to foreign antigens with a cascade of molecular and cellular events that ultimately results in the humoral and cell-mediated immune response.

The major pathway of the immune defense involving antigen-specific recognition commences with the trapping of the antigen by antigen presenting cells (APCs), such as dendritic cells or macrophages, and the subsequent migration of these cells to lymphoid organs (e.g., thymus). There, the APCs present antigen to subclasses of T cells classified as mature T helper cells. Upon specific recognition of the presented antigen, the mature T helper cells can be triggered to become activated T helper cells. The activated T helper cells regulate both the humoral immune response by inducing the differentiation of mature B cells to antibody producing plasma cells and the cell-mediated immune response by activation of mature cytotoxic T cells.

The thymus has been shown to be an obligatory factor in T cell differentiation of hematopoietic cells. Based upon the murine model, it is believed that the presence of a three dimensional organ is required, as *in vitro* models that do not include the thymus and a three dimensional structure fail to support T cell lymphopoiesis (Owen JJ, et al., *Br Med Bull.*,

1989, 45:350-360). The process of differentiation, however, appears to begin prior to progenitor cells contacting the thymus.

Primitive hematopoietic progenitors in the fetal liver or bone marrow give rise to lineage committed cells, including progenitors committed to the T lymphoid lineage. These most immature cells are identified by the surface expression of CD34. T cell lineage committed cells express CD34, but no discrete expression of other epitopes found only on T cell progenitors has been described. Further, T lymphocyte differentiation normally occurs via a series of discrete developmental stages. Primitive progenitor cells which do not express lymphocyte specific cell surface markers (CD34+ CD3- CD4- CD8-) migrate to the thymus where they acquire, through a series of maturational events, the phenotype CD34- CD3- CD4+ CD8-. These cells then mature into double positive CD4+ CD8+ cells, most of which are CD3+, although CD3 expression is not universally detectable. These cells further undergo both positive and negative selection, and mature to develop into single positive T cells (CD4+ CD8- or CD4- CD8+). These cells ultimately migrate into the peripheral circulation as naive T cells.

T cell disorders and diseases represent major health problems. Recent progress has been made using gene therapy to treat conditions involving T lymphocytes, including AIDS. This has fostered increased interest in the development of laboratory techniques that allow *in vitro* evaluations of potential genetic therapies for these conditions.

The understanding of T cell differentiation has been hampered by the limited availability of technologies which permit *in vitro* T cell differentiation. To date, T cell differentiation studies have been largely confined to the SCID-hu mouse *in vivo* model. *In vitro* technologies have been based on thymic explant studies and primate thymic monolayers. In a recent advance, primate thymic stroma cultures have been shown to provide an expedient, although inefficient, system for examining T cell development, enabling *in vitro* T cell differentiation in a reproducible manner. However, the purity and number of T cells generated this way, as well as the relatively short half-life of the cultures, generally results in limited applicability to more advanced studies of T cell differentiation and function.

Summary of the Invention

The invention, in one important part, involves improved methods for culturing hematopoietic progenitor cells that direct their development toward lymphoid tissue-specific lineages without the addition of exogenous growth factors. Thus, one aspect of the invention is the culture of hematopoietic progenitor cells to generate progeny committed to a specific

lineage. Another aspect is an improvement in the rate and the number of differentiated progeny that can be obtained from a sample of hematopoietic progenitor cells.

We describe herein a system that takes advantage of biocompatible, open-pore, three-dimensional matrices, and uses human and non-human lymphoreticular stromal cells to provide the appropriate conditions for the expansion and differentiation of human and non-human hematopoietic progenitor cells toward a specific cell lineage. T lymphocytes, for example, derived from these cultures respond normally to a variety of stimuli and express the diversity of markers expected of mature T cells.

This system provides significant advantages over existing techniques. For example, it can provide for the rapid generation of a large number of differentiated progeny necessary for laboratory analysis and/or therapeutic uses, including for *in vitro* testing of potential gene therapy strategies or for reinfusion into subjects *in vivo*. The matrix itself can be implanted into subjects for *in vivo* studies of hematopoietic cell growth. The system also can reasonably replicate the complex process of hematopoietic cell maintenance, expansion and/or differentiation toward a specific lineage.

Surprisingly, according to the invention, it has been discovered that hematopoietic progenitor cells co-cultured with lymphoreticular stromal cells in a porous solid scaffold, without the addition of exogenous growth agents, generate at a fast rate an unexpectedly high number of functional, differentiated progeny of a lymphoid-specific lineage. The lymphoid tissue from which lymphoreticular stromal cells are derived helps determine the lineage-commitment hematopoietic progenitor cells undertake, resulting in the lineage-specificity of the differentiated progeny. Also surprising, according to the invention, is the discovery that lesser amounts of nonlymphoid cells (i.e. myelo-monocytic cells) are generated from the co-culture of hematopoietic progenitor cells and lymphoreticular stromal cells in a porous solid scaffold of the invention when compared to existing methodology. Thus, the present invention permits for the rapid generation of a large number of differentiated, lymphoid-specific cells from a relatively small number of hematopoietic progenitor cells. Such results were never before realized using known art methodologies (e.g., as in U.S. Patent No. 5,677,139 by Johnson *et al.*, which describes the *in vitro* differentiation of CD3⁺ cells on primate thymic stroma monolayers, or as in U.S. Patent No. 5,541,107 by Naughton *et al.*, which describes a three-dimensional bone marrow cell and tissue culture system).

According to one aspect of the invention, a method for *in vitro* production of lymphoid tissue-specific cells is provided. The method involves introducing an amount of

hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix. The hematopoietic progenitor cells and the lymphoreticular stromal cells are then co-cultured. The amount of the lymphoreticular stromal cells utilized is sufficient to support the growth and differentiation of the hematopoietic progenitor cells. In one embodiment, co-culturing occurs under conditions sufficient to produce at least a 10-fold increase in the number of lymphoid tissue origin cells. In preferred embodiments, co-culturing occurs under conditions sufficient to produce at least a 20, 50, 100, 200, 300 or 400 -fold increase in the number of lymphoid tissue origin cells. In some embodiments, after the co-culturing, harvesting of the lymphoid tissue origin cells may be performed.

In certain embodiments, the hematopoietic progenitor cells may be pluripotent stem cells, multipotent progenitor cells and/or progenitor cells committed to specific hematopoietic lineages. The progenitor cells committed to specific hematopoietic lineages may be of T cell lineage, B cell lineage, dendritic cell lineage, Langerhans cell lineage and/or lymphoid tissue-specific macrophage cell lineage.

The hematopoietic progenitor cells may be derived from a tissue such as bone marrow, peripheral blood (including mobilized peripheral blood), umbilical cord blood, placental blood, fetal liver, embryonic cells (including embryonic stem cells), aortal-gonadal-mesonephros derived cells, and lymphoid soft tissue. Lymphoid soft tissue includes the thymus, spleen, liver, lymph node, skin, tonsil and Peyer's patches. In other embodiments, the lymphoreticular stromal cells may be also derived from at least one of the foregoing lymphoid soft tissues. In preferred embodiments, the lymphoreticular stromal cells are thymic stromal cells and the multipotent progenitor cells and/or committed progenitor cells are committed to a T cell lineage. In other embodiments, the hematopoietic progenitor cells and/or the lymphoreticular stromal cells may be genetically altered.

In one important embodiment of the invention, the hematopoietic progenitor cells are of human origin and the lymphoreticular stromal cells are also of human origin. In another embodiment, the hematopoietic progenitor cells are of human origin and the lymphoreticular stromal cells are of non-human origin. In preferred embodiments, non-human lymphoreticular stromal cells are of murine origin.

In certain embodiments, the lymphoreticular stromal cells are seeded to the matrix at the same time as the hematopoietic progenitor cells. In other embodiments, the

lymphoreticular stromal cells are seeded to the matrix prior to inoculating the hematopoietic progenitor cells.

The porous matrix can be one that is an open cell porous matrix having a percent open space of at least 50%, and preferably at least 75%. In one embodiment the porous solid
5 matrix has pores defined by interconnecting ligaments having a diameter at midpoint, on average, of less than 150 μm . Preferably the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material, the metal coating being selected from the group consisting of tantalum, titanium, platinum (including other metals of the platinum group), niobium, hafnium, tungsten, and combinations thereof. In preferred embodiments, whether
10 the porous solid matrix is metal-coated or not, the matrix is coated with a biological agent selected from the group consisting of collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitronectin, antibodies and fragments thereof, functional equivalents of these factors (including fragments thereof), and combinations thereof. Most preferably the metal coating is tantalum coated with a biological
15 agent. In certain other embodiments, the porous solid matrix having seeded hematopoietic progenitor cells and their progeny, and lymphoreticular stromal cells, is impregnated with a gelatinous agent that occupies pores of the matrix.

The preferred embodiments of the invention are solid, unitary macrostructures, i.e. not beads or packed beads. They also involve nonbiodegradable materials.

20 According to any of the foregoing embodiments, the method of the invention can include culturing the cells in an environment that is free of hematopoietic progenitor cell survival and proliferation factors such as interleukins 3, 6 and 11, stem cell ligand and FLT-3 ligand. Still another embodiment of the invention is performing the co-culturing of the hematopoietic progenitor cells and the lymphoreticular stromal cells in an environment that is
25 free altogether of stromal cell conditioned medium and exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion and/or differentiation, other than serum.

As will be understood, according to the invention, it is possible now to co-culture hematopoietic progenitor cells and lymphoreticular stromal cells in an environment that is
30 free of exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion and/or differentiation for as little as 7 days and to obtain large numbers of differentiated progeny of a specific lineage.

According to any of the foregoing embodiments, the method of the invention can include co-culturing of the hematopoietic progenitor cells and the lymphoreticular stromal cells with an exogenously added agent selected from the group consisting of stromal cell conditioned medium, and a hematopoietic growth factor that promotes hematopoietic cell maintenance, expansion and/or differentiation, and influences cell localization. In certain
5 embodiments, the hematopoietic growth factor that promotes hematopoietic cell maintenance, expansion and/or differentiation, and influences cell localization, may be an agent that includes interleukin 3, interleukin 6, interleukin 7, interleukin 11, interleukin 12, stem cell factor, FLK-2 ligand, FLT-2 ligand, Epo, Tpo, GMCSF, GCSF, Oncostatin M, and MCSF.

10 According to another aspect of the invention, a method for *in vivo* maintenance, expansion and/or differentiation of hematopoietic progenitor cells is provided. The method involves implanting into a subject a porous, solid matrix having seeded therein hematopoietic progenitor cells (which may include their progeny) and lymphoreticular stromal cells. The porous matrix has interconnected pores of a pore size sufficient to permit the cells to grow
15 throughout the matrix and is an open cell porous matrix having a percent open space of at least 50%, and preferably at least 75%. Various embodiments are provided, wherein the porous solid matrix has one or more of the preferred characteristics as described above.

In certain embodiments, hematopoietic progenitor cells (that may include progeny) and lymphoreticular stromal cells are attached to the matrix by introducing *in vitro* an amount
20 of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into the porous solid matrix, and co-culturing the hematopoietic progenitor cells in an environment that is free of stromal cell conditioned medium and free of exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion and/or differentiation, other than serum. Various other embodiments are provided, wherein the co-culturing is
25 performed under conditions as described above. In yet other embodiments, the porous solid matrix having seeded hematopoietic progenitor cells (that may include progeny) and lymphoreticular stromal cells is impregnated with a gelatinous agent that occupies pores of the matrix.

According to one aspect of the invention, a method for inducing T cell anergy *in vitro*
30 is provided. The method involves introducing an amount of hematopoietic progenitor cells, an amount of antigen presenting cells, and an amount of lymphoreticular stromal cells into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the

matrix, and co-culturing the hematopoietic progenitor cells, the antigen presenting cells and the lymphoreticular stromal cells in the presence of at least one antigen under conditions sufficient to induce the formation of T cells and/or T cell progenitors and to inhibit activation of the formed T cells and/or T cell progenitors.

5 In certain embodiments, the hematopoietic progenitor cells may be pluripotent stem cells, multipotent progenitor cells and/or progenitor cells committed to specific hematopoietic lineages. The hematopoietic progenitor cells may be derived from a tissue such as bone marrow, peripheral blood (including mobilized peripheral blood), umbilical cord blood, placental blood, fetal liver, embryonic cells (including embryonic stem cells), aortal-gonadal-
10 mesonephros derived cells, and lymphoid soft tissue. Lymphoid soft tissue includes the thymus, spleen, liver, lymph node, skin, tonsil and/or Peyer's patches. In other embodiments, the lymphoreticular stromal cells may be also derived from at least one of the foregoing lymphoid soft tissues. In preferred embodiments, the lymphoreticular stromal cells are thymic stromal cells and the multipotent progenitor cells and/or committed progenitor cells
15 are committed to a T cell lineage. In other embodiments, the hematopoietic progenitor cells and/or the lymphoreticular stromal cells may be genetically altered. In certain embodiments, the antigen presenting cells include cells such as dendritic cells, monocytes/macrophages, Langerhans cells, Kupfer cells, microglia, alveolar macrophages and B cells. In other embodiments, the antigen presenting cells are derived from hematopoietic progenitor cells *in*
20 *vitro*. Various embodiments are provided, wherein the porous solid matrix has one or more of the preferred characteristics as described above.

According to another aspect of the invention, a method for inducing T cell reactivity *in vitro* is provided. The method involves introducing an amount of hematopoietic progenitor cells, an amount of antigen presenting cells, and an amount of lymphoreticular stromal cells
25 into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix, and co-culturing the hematopoietic progenitor cells, the antigen presenting cells and the lymphoreticular stromal cells in the presence of at least one antigen under conditions sufficient to induce the formation of T cells and/or T cell progenitors from the hematopoietic
30 progenitor cells having specificity for the at least one antigen. Various embodiments are provided, wherein the hematopoietic progenitor cells, the lymphoreticular stromal cells, and the porous solid matrix have one or more of the preferred characteristics as described above, and the cells are cultured as described above. In certain embodiments, the antigen presenting

cells include cells such as dendritic cells, monocytes/macrophages, Langerhans cells, Kupfer cells, microglia, alveolar macrophages and B cells. In other embodiments, the antigen presenting cells are derived from hematopoietic progenitor cells *in vitro*. In further
5 culture. Preferred co-stimulatory agents include lymphocyte function associated antigen 3 (LFA-3), CD2, CD40, CD80/B7-1, CD86/B7-2, OX-2, CD70, and CD82.

In yet another aspect of the invention, a solid porous matrix is provided wherein hematopoietic progenitor cells, with or without their progeny, and lymphoreticular stromal cells are attached to the solid porous matrix. The lymphoreticular stromal cells are present in
10 an amount sufficient to support the growth and differentiation of hematopoietic progenitor cells. In certain embodiments, the hematopoietic progenitor cells are attached to the lymphoreticular stromal cells. The porous matrix can be one that is an open cell porous matrix having a percent open space of at least 50%, and preferably at least 75%. In one embodiment the porous solid matrix has pores defined by interconnecting ligaments having a diameter at
15 midpoint, on average, of less than 150 μm . Preferably the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material, the metal coating being selected from the group consisting of tantalum, titanium, platinum (including other metals of the platinum group), niobium, hafnium, tungsten, and combinations thereof. In preferred
20 embodiments, whether the porous solid matrix is metal-coated or not, the matrix is coated with a biological agent selected from the group consisting of collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitrogen, antibodies and fragments thereof, functional equivalents of these factors, and combinations thereof. Most preferably the metal coating is tantalum coated with a biological agent. In
25 certain other embodiments the porous solid matrix having seeded hematopoietic progenitor cells and lymphoreticular stromal cells, is impregnated with a gelatinous agent that occupies pores of the matrix.

In a further aspect of the invention, a method for identifying an agent suspected of affecting hematopoietic cell development, is provided. The method involves introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into
30 a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix, co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells in the presence of at least one candidate agent suspected of affecting hematopoietic cell

development (in a test co-culture), and determining whether the at least one candidate agent affects hematopoietic cell development in the test co-culture by comparing the test co-culture hematopoietic cell development to a control co-culture, whereby hematopoietic progenitor cells and lymphoreticular stromal cells are co-cultured in the absence of the at least one candidate agent. Various embodiments are provided, wherein the hematopoietic progenitor cells, the lymphoreticular stromal cells, and the porous solid matrix have one or more of the preferred characteristics as described above, and the cells are cultured as described above. In certain embodiments, hematopoietic progenitor cell development includes hematopoietic progenitor cell maintenance, expansion, differentiation toward a specific cell lineage, and/or cell-death (including apoptosis). In preferred embodiments the lymphoreticular stromal cells are thymic stromal cells.

In another aspect of the invention, a method for isolating from a cell culture an agent suspected of affecting hematopoietic cell development, is provided. The method involves introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix, co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells, obtaining a test-supernatant from the co-culture, comparing the test-supernatant to a control-supernatant, and obtaining a subfraction of the test-supernatant that contains an agent suspected of affecting hematopoietic cell development that is absent from the control-supernatant. In certain embodiments the agent suspected of affecting hematopoietic cell development may be present in the control-supernatant and absent from the test-supernatant. In other embodiments, the agent suspected of affecting hematopoietic cell development in one supernatant may be different to an agent suspected of affecting hematopoietic cell development in the other supernatant (e.g., in size, via a post-translational modification, in an alternatively spliced variant form, etc.). Various embodiments are provided, wherein the hematopoietic progenitor cells, the lymphoreticular stromal cells, and the porous solid matrix have one or more of the preferred characteristics as described above, and the cells are cultured as described above. In certain embodiments, hematopoietic progenitor cell development includes hematopoietic progenitor cell maintenance, expansion, differentiation toward a specific cell lineage, and/or cell-death (including apoptosis). In preferred embodiments, the lymphoreticular stromal cells are thymic stromal cells. In certain other embodiments, the control culture system of the prior art

(where the control-supernatant can be obtained from) is the one described in U.S. Patent No. 5,677,139 by Johnson *et al.*

These and other aspects of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments.

Brief Description of the Drawings

Figure 1 shows the differentiation of human CD34⁺ progenitor cells into T cells, in co-culture with murine thymic stroma cells on a three-dimensional matrix; the data in Fig. 1(a) shows the acquisition of CD2 and the down-regulation of the hematopoietic progenitor cell marker CD34; the data in Fig. 1(b) shows the discrete populations of SP CD4⁺ and SP CD8⁺ cells, including their DP CD4⁺CD8⁺ precursors; the data in Figs. 1(c and d) shows that all CD4⁺(c) and CD8⁺(d) cells co-expressed CD3.

Figure 2 shows the intrasample variability in numbers of T cells generated in a co-culture system of the invention.

Figure 3 shows the intersample variability in numbers of T cells generated in a co-culture system of the invention.

Detailed Description of the Invention

The invention involves the unexpected discovery that hematopoietic progenitor cells co-cultured with lymphoreticular stromal cells in a porous solid scaffold, without the addition of exogenous growth agents, generate at a fast rate an unexpectedly high number of functional, differentiated progeny of a lymphoid tissue-specific lineage. Also surprising, according to the invention, has been the discovery that lesser amounts of nonlymphoid cells (i.e. myelo-monocytic cells) are generated from the co-culture of hematopoietic progenitor cells and lymphoreticular stromal cells in a porous solid scaffold of the invention when compared to existing technology. Thus, the present invention, and in contrast to what has been previously achieved in the art, permits for the rapid generation of a large number of differentiated, lymphoid-specific cells from a relatively small number of hematopoietic progenitor cells.

Methods of the invention are therefore useful *inter alia* for establishing immunocompetence in patients suffering from an immunodeficiency, e.g., a T cell or B cell deficiency, e.g., a thymic based immunodeficiency, e.g., a congenital immunodeficiency due to thymic aplasia or dysfunction, an acquired immune disorder, e.g., AIDS, immunoincompetence resulting from a neoplastic disease, or immunoincompetence resulting

from a medical procedure, e.g., chemotherapy, immunocompetence in response to an antigen, etc.

The invention in one aspect involves culturing hematopoietic cells in a porous solid matrix, in the absence of exogenous growth agents, to produce lymphoid tissue origin (lymphoid tissue-specific) cells.

A porous, solid matrix, is defined as a three-dimensional structure with "sponge-like" continuous pores forming an interconnecting network. The matrix can be rigid or elastic, and it provides a scaffold upon which cells can grow throughout. Its pores are interconnected and provide the continuous network of channels extending through the matrix and also permit the flow of nutrients throughout. A preferred matrix is an open cell foam matrix having a percent open space of at least 50% and preferably 75%. Thus, it is preferred that the open space comprise the majority of the matrix. This is believed to maximize cell migration, cell-cell contact, space for cell growth and accessibility to nutrients. It is preferred that the porous matrix be formed of a reticulated matrix of ligaments which at their center point are less than 150 μ m in diameter, preferably 60 μ m, whereby a cell can reside on or interact with a portion of the ligament. Preferably, the average pore diameter is on the order of 300 μ m, which resembles cancellous bone. Suitable matrices can be obtained using a number of different methods. Examples of such methods include solvent casting or extraction of polymers, track etching of a variety of materials, foaming of a polymer, the replamineform process for hydroxyapatite, and other methodologies well known to those of ordinary skill in the art. The materials employed can be natural or synthetic, including biological materials such as proteins, hyaluronic acids, synthetic polymers such as polyvinyl pyrrolidones, polymethylmethacrylate, methyl cellulose, polystyrene, polypropylene, polyurethane, ceramics such as tricalcium phosphate, calcium aluminate, calcium hydroxyapatite and ceramic-reinforced or coated polymers. If the starting material for the scaffold is not metal, a metal coating can be applied to the three-dimensional matrix. Metal coatings provide further structural support and/or cell growth and adhesive properties to the matrix. Preferred metals used as coatings comprise tantalum, titanium, platinum and metals in the same element group as platinum, niobium, hafnium, tungsten, and combinations of alloys thereof. Coating methods for metals include a process such as CVD (Chemical Vapor Deposition).

The preferred matrix, referred to herein throughout as Cellfoam (Cytomatrix, Woburn, MA), is described in detail in U.S. Patent No. 5,282,861, and is incorporated herein by reference. More specifically, the preferred matrix is a reticulated open cell substrate formed

by a lightweight, substantially rigid foam of carbon-containing material having open spaces defined by an interconnecting network, wherein said foam material has interconnected continuous channels, and a thin film of metallic material deposited onto the reticulated open cell substrate and covering substantially all of the interconnecting network to form a composite porous biocompatible material creating a porous microstructure similar to that of natural cancellous bone.

Additionally, such matrices can be coated with biological agents which can promote cell adhesion for the cultured hematopoietic progenitor cells, allowing for improved migration, growth and proliferation. Moreover, when these matrices are used for the *in vivo* maintenance, expansion and/or differentiation of hematopoietic progenitor cells (i.e., when the matrices with the cells are implanted into a subject, -see also discussion below), biological agents that promote angiogenesis (vascularization) and biological agents that prevent/reduce inflammation may also be used for coating of the matrices. Preferred biological agents comprise collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitrogen, antibodies and fragments thereof, functional equivalents of these agents, and combinations thereof.

Angiogenic factors include platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), bFGF-2, leptins, plasminogen activators (tPA, uPA), angiopoietins, lipoprotein A, transforming growth factor- β , bradykinin, angiogenic oligosaccharides (e.g., hyaluronan, heparan sulphate), thrombospondin, hepatocyte growth factor (also known as scatter factor) and members of the CXC chemokine receptor family. Anti-inflammatory factors comprise steroidal and non-steroidal compounds and examples include: Alclofenac; Alclometasone Dipropionate; Algestone Acetonide; Alpha Amylase; Amcinafal; Amcinafide; Amfenac Sodium; Amiprilose Hydrochloride; Anakinra; Anirolac; Anitrazafen; Apazone; Balsalazide Disodium; Bendazac; Benoxaprofen; Benzydamine Hydrochloride; Bromelains; Broperamole; Budesonide; Carprofen; Cicloprofen; Cintazone; Cliprofen; Clobetasol Propionate; Clobetasone Butyrate; Clopirac; Cloticasone Propionate; Cormethasone Acetate; Cortodoxone; Deflazacort; Desonide; Desoximetasone; Dexamethasone Dipropionate; Diclofenac Potassium; Diclofenac Sodium; Diflorasone Diacetate; Diflumidone Sodium; Diflunisal; Difluprednate; Diftalone; Dimethyl Sulfoxide; Drocinnonide; Endrysone; Enlimomab; Enolicam Sodium; Epirizole; Etodolac; Etofenamate; Felbinac; Fenamole; Fenbufen; Fenclofenac; Fenclorac; Fendosal; Fenpipalone; Fentiazac; Flazalone; Fluazacort; Flufenamic Acid; Flumizole; Flunisolid

Acetate; Flunixin; Flunixin Meglumine; Fluocortin Butyl; Fluorometholone Acetate; Fluquazone; Flurbiprofen; Fluretofen; Fluticasone Propionate; Furaprofen; Furobufen; Halcinonide; Halobetasol Propionate; Halopredone Acetate; Ibufenac; Ibuprofen; Ibuprofen Aluminum; Ibuprofen Piconol; Ilonidap; Indomethacin; Indomethacin Sodium; Indoprofen; 5 Indoxole; Intrazole; Isoflupredone Acetate; Isoxepac; Isoxicam; Ketoprofen; Lofemizole Hydrochloride; Lornoxicam; Loteprednol Etabonate; Meclofenamate Sodium; Meclofenamic Acid; Meclorison Dibutyrate; Mefenamic Acid; Mesalamine; Meseclazone; Methylprednisolone Suleptanate; Morniflumate; Nabumetone; Naproxen; Naproxen Sodium; Naproxol; Nimazone; Olsalazine Sodium; Orgotein; Orpanoxin; Oxaprozin; 10 Oxyphenbutazone; Paranyline Hydrochloride; Pentosan Polysulfate Sodium; Phenbutazone Sodium Glycerate; Pirfenidone; Piroxicam; Piroxicam Cinnamate; Piroxicam Olamine; Pirprofen; Prednazate; Prifelone; Prodolic Acid; Proquazone; Proxazole; Proxazole Citrate ; Rimexolone; Romazarit ; Salcolex ; Salnacedin; Salsalate ; Sanguinarium Chloride ; Seclazone ; Sermetacin; Sudoxicam; Sulindac; Suprofen; Talmetacin; Talniflumate; 15 Talosalate; Tebufelone; Tenidap; Tenidap Sodium; Tenoxicam; Tesicam; Tesimide; Tetrydamine ; Tiopinac ; Tixocortol Pivalate; Tolmetin; Tolmetin Sodium; Triclonide; Triflumidate; Zidometacin; Zomepirac Sodium.

In certain embodiments of the invention the porous solid matrix having seeded hematopoietic progenitor cells, with or without their progeny, and lymphoreticular stromal 20 cells is impregnated with a gelatinous agent that occupies pores of the matrix. The hematopoietic progenitor cells, with or without their progeny, and/or the lymphoreticular stromal cells can be seeded prior to, substantially at the same time as, or following impregnation (or infiltration) with a gelatinous agent. For example, the cells may be mixed with the agent and seeded at the same time as the the impregnation of the matrix with the 25 agent. In some embodiments, the cells are seeded onto the porous solid matrix prior to application of the agent. In certain embodiments the lymphoreticular stromal cells are seeded in a similar manner. A person of ordinary skill in the art can easily determine seeding conditions. Preferably the lymphoreticular stromal cells are seeded prior to the hematopoietic progenitor cells and prior to impregnation with the agent.

30 “Impregnation” with a gelatinous agent can serve, *inter alia*, to contain the cells within the matrix, or to help maintain and/or enhance cell attachment onto the matrix. The “gelatinous” agent may be one that can be maintained in a fluid state initially (i.e. gelable), and after its application into the matrix, be gelatinized *in situ* in the matrix. Such

gelatinization may occur in a number of different ways, including altering the agent's temperature, irradiating the agent with an energy source (e.g., light), etc. The "gelatinous" agent also is characterized by its ability to allow the nutrients of the growth media to reach the cells throughout the matrix. Exemplary "gelatinous" agents include cellulosic polysaccharides (such as cellulose, hemicellulose, methylcellulose, and the like), agar, agarose, albumin, algal mucin, mucin, mucilage, collagens, glycosaminoglycans, and proteoglycans (including their sulphated forms). In certain embodiments, the gelatinous agent may impregnate the matrix completely, in some embodiments partially, and in other embodiments minimally, serving only as a coating of all or some of the outer surfaces of the matrix. In important embodiments where gelatinous agents are employed, the "gelatinous" agent is methylcellulose and the impregnation is complete.

According to the invention, hematopoietic progenitor cells and lymphoreticular stromal cells are co-cultured in one of the foregoing porous solid matrices, in the absence of exogenous growth agents, to produce lymphoid tissue origin (lymphoid tissue-specific) cells. "Lymphoid tissue origin" (lymphoid tissue-specific) cells, as used herein, refer to cells that may be produced *in vitro* or *in vivo* according to the invention, and are substantially similar (e.g., in properties and function) to the cells produced naturally *in vivo* from organs and tissues that include the bone marrow, thymus, lymph nodes, spleen and mucosal associated lymphoid tissue (unencapsulated tissue lining the respiratory, alimentary and genito-urinary tracts).

"Hematopoietic progenitor cells" as used herein refers to immature blood cells having the capacity to self-renew and to differentiate into the more mature blood cells (also described herein as "progeny") comprising granulocytes (e.g., promyelocytes, neutrophils, eosinophils, basophils), erythrocytes (e.g., reticulocytes, erythrocytes), thrombocytes (e.g., megakaryoblasts, platelet producing megakaryocytes, platelets), and monocytes (e.g., monocytes, macrophages). It is known in the art that such cells may or may not include CD34⁺ cells. CD34⁺ cells are immature cells present in the "blood products" described below, express the CD34 cell surface marker, and are believed to include a subpopulation of cells with the "progenitor cell" properties defined above. It is well known in the art that hematopoietic progenitor cells include pluripotent stem cells, multipotent progenitor cells (e.g., a lymphoid stem cell), and/or progenitor cells committed to specific hematopoietic lineages. The progenitor cells committed to specific hematopoietic lineages may be of T cell

lineage, B cell lineage, dendritic cell lineage, Langerhans cell lineage and/or lymphoid tissue-specific macrophage cell lineage.

The hematopoietic progenitor cells can be obtained from blood products. A “blood product” as used in the present invention defines a product obtained from the body or an organ of the body containing cells of hematopoietic origin. Such sources include unfractionated bone marrow, umbilical cord, peripheral blood, liver, thymus, lymph and spleen. It will be apparent to those of ordinary skill in the art that all of the aforementioned crude or unfractionated blood products can be enriched for cells having “hematopoietic progenitor cell” characteristics in a number of ways. For example, the blood product can be depleted from the more differentiated progeny. The more mature, differentiated cells can be selected against, via cell surface molecules they express. Additionally, the blood product can be fractionated selecting for CD34⁺ cells. As mentioned earlier, CD34⁺ cells are thought in the art to include a subpopulation of cells capable of self-renewal and pluripotentiality. Such selection can be accomplished using, for example, commercially available magnetic anti-CD34 beads (Dynal, Lake Success, NY). Unfractionated blood products can be obtained directly from a donor or retrieved from cryopreservative storage.

The cells co-cultured with the hematopoietic progenitor cells according to the methods of the invention are lymphoreticular stromal cells. “Lymphoreticular stromal cells” as used herein may include, but are not limited to, all cell types present in a lymphoid tissue which are not lymphocytes or lymphocyte precursors or progenitors, e.g., epithelial cells, endothelial cells, mesothelial cells, dendritic cells, splenocytes and macrophages. Lymphoreticular stromal cells also include cells that would not ordinarily function as lymphoreticular stromal cells, such as fibroblasts, which have been genetically altered to secrete or express on their cell surface the factors necessary for the maintenance, growth and/or differentiation of hematopoietic progenitor cells, including their progeny. Lymphoreticular stromal cells are derived from the disaggregation of a piece of lymphoid tissue (see discussion below and the Examples). Such cells according to the invention are capable of supporting *in vitro* the maintenance, growth and/or differentiation of hematopoietic progenitor cells, including their progeny. By “lymphoid tissue” it is meant to include bone marrow, peripheral blood (including mobilized peripheral blood), umbilical cord blood, placental blood, fetal liver, embryonic cells (including embryonic stem cells), aortal-gonadal-mesonephros derived cells, and lymphoid soft tissue. “Lymphoid soft tissue” as used herein includes, but is not limited

to, tissues such as thymus, spleen, liver, lymph node, skin, tonsil, adenoids and Peyer's patch, and combinations thereof.

Lymphoreticular stromal cells provide the supporting microenvironment in the intact lymphoid tissue for the maintenance, growth and/or differentiation of hematopoietic progenitor cells, including their progeny. The microenvironment includes soluble and cell surface factors expressed by the various cell types which comprise the lymphoreticular stroma. Generally, the support which the lymphoreticular stromal cells provide may be characterized as both contact-dependent and non-contact-dependent.

Lymphoreticular stromal cells may be allogeneic, syngeneic or xenogeneic with respect to the hematopoietic progenitor cells. Lymphoreticular stroma cells may be obtained from the lymphoid tissue of a human or a non-human subject at any time after the organ/tissue has developed to a stage (i.e., the maturation stage) at which it can support the maintenance growth and/or differentiation of hematopoietic progenitor cells. The stage will vary between organs/tissues and between subjects. In primates, for example, the maturation stage of thymic development is achieved during the second trimester. At this stage of development the thymus can produce peptide hormones such as thymulin, α_1 and β_4 -thymosin, and thymopoietin, as well as other factors required to provide the proper microenvironment for T cell differentiation. The different maturation stages for the different organs/tissues and between different subjects are well known in the art.

The lymphoid tissue from which lymphoreticular stromal cells are derived usually determines the lineage-commitment hematopoietic progenitor cells undertake, resulting in the lineage-specificity of the differentiated progeny. In certain embodiments, the lymphoreticular stromal cells are thymic stromal cells and the multipotent progenitor cells and/or committed progenitor cells are committed to a T cell lineage. In other embodiments, the lymphoreticular stromal cells may be splenic stromal cells and the multipotent progenitor cells and/or committed progenitor cells are committed to a B cell lineage. Also surprising, according to the invention, has been the discovery that the highest yield of differentiated progeny occurs when human hematopoietic progenitor cells are cultured in the presence of xenogeneic (non-human) lymphoreticular stromal cells. Preferably the xenogeneic lymphoreticular stromal cells are of murine origin.

Unexpectedly, it has also been discovered that lesser amounts of nonlymphoid-specific cells (i.e. myelo-monocytic cells) are generated from the foregoing co-cultures when compared to existing methodology. In other words, more homogeneous differentiation of

cells with fewer contaminant cell types (nonlymphoid) is observed from cultures of the present invention on Cellfoam, enabling the preservation of immature progenitors (CD34⁺ cells) while promoting the differentiation of more mature T progeny.

Various other embodiments are provided, wherein the lymphoreticular stromal cells may be genetically altered. The lymphoreticular stromal cells may be transfected with exogenous DNA that encodes, for example, one of the hematopoietic growth factors described above (see fibroblast discussion above).

As mentioned earlier, lymphoreticular stromal cells are derived from the disaggregation of a piece of lymphoid tissue, forming cell suspensions. Preferably, single cell suspensions are generated. These lymphoreticular stromal cell suspensions may be used directly, or made non-mitotic by procedures standard in the tissue culture art. Examples of such methods are irradiation of lymphoreticular stromal cells with a gamma-ray source or incubation of the cells with mitomycin C for a sufficient amount of time to render the cells mitotically inactive. Mitotic inactivation is preferred when the lymphoreticular stromal cells are of human origin (to eliminate progenitor cells that may be present in the suspension). The lymphoreticular stromal cells may then be seeded into a three-dimensional matrix of the invention and permitted to attach to a surface of the porous, solid matrix. It should be noted that the lymphoreticular stromal cells may alternatively be cryopreserved for later use or for storage and shipment to remote locations, such as for use in connection with the sale of kits. Cryopreservation of cells cultured *in vitro* is well established in the art. Subsequent to isolation (and/or mitotic inactivation) of a cell sample, cells may be cryopreserved by first suspending the cells in a cryopreservation medium and then gradually freezing the cell suspension. Frozen cells are typically stored in liquid nitrogen or at an equivalent temperature in a medium containing serum and a cryopreservative such as dimethyl sulfoxide.

The co-culture of the hematopoietic progenitor cells (and progeny thereof) with lymphoreticular stromal cells, preferably occurs under conditions sufficient to produce a percent increase in the number of lymphoid tissue origin cells deriving from the hematopoietic progenitor cells. The conditions used refer to a combination of conditions known in the art (e.g., temperature, CO₂ and O₂ content, nutritive media, time-length, etc.).

The time sufficient to increase the number of cells is a time that can be easily determined by a person skilled in the art, and can vary depending upon the original number of cells seeded. The amounts of hematopoietic progenitor cells and lymphoreticular stromal cells initially introduced (and subsequently seeded) into the porous solid matrix may vary according to the

needs of the experiment. The ideal amounts can be easily determined by a person skilled in the art in accordance with needs. Preferably, the lymphoreticular stromal cells would form a confluent layer onto the matrix. Hematopoietic progenitor cells may be added at different numbers. As an example, discoloration of the media over a certain period of time can be used
5 as an indicator of confluency. Additionally, and more precisely, different numbers of hematopoietic progenitor cells or volumes of the blood product can be cultured under identical conditions, and cells can be harvested and counted over regular time intervals, thus generating the "control curves". These "control curves" can be used to estimate cell numbers in subsequent occasions (see the Examples section).

10 The conditions for determining colony forming potential are similarly determined. Colony forming potential is the ability of a cell to form progeny. Assays for this are well known to those of ordinary skill in the art and include seeding cells into a semi-solid matrix, treating them with growth factors, and counting the number of colonies.

In preferred embodiments of the invention, the hematopoietic progenitor cells may be
15 harvested. "Harvesting" hematopoietic progenitor cells is defined as the dislodging or separation of cells from the matrix. This can be accomplished using a number of methods, such as enzymatic and non-enzymatic, centrifugal, electrical or by size, or the one preferred in the present invention, by flushing of the cells using the media in which the cells are incubated. The cells can be further collected, separated, and further expanded generating even larger
20 populations of differentiated progeny.

As mentioned above, the hematopoietic progenitor cells, and progeny thereof, can be genetically altered. Genetic alteration of a hematopoietic progenitor cell includes all transient and stable changes of the cellular genetic material which are created by the addition of exogenous genetic material. Examples of genetic alterations include any gene therapy
25 procedure, such as introduction of a functional gene to replace a mutated or nonexpressed gene, introduction of a vector that encodes a dominant negative gene product, introduction of a vector engineered to express a ribozyme and introduction of a gene that encodes a therapeutic gene product. Natural genetic changes such as the spontaneous rearrangement of a T cell receptor gene without the introduction of any agents are not included in this concept.
30 Exogenous genetic material includes nucleic acids or oligonucleotides, either natural or synthetic, that are introduced into the hematopoietic progenitor cells. The exogenous genetic material may be a copy of that which is naturally present in the cells, or it may not be

naturally found in the cells. It typically is at least a portion of a naturally occurring gene which has been placed under operable control of a promoter in a vector construct.

The invention involves the unexpected discovery that hematopoietic progenitor cells can be more efficiently genetically altered if the genetic alteration occurs while the hematopoietic progenitor cells are on and within a solid porous matrix as described above.

Various techniques may be employed for introducing nucleic acids into cells. Such techniques include transfection of nucleic acid-CaPO₄ precipitates, transfection of nucleic acids associated with DEAE, transfection with a retrovirus including the nucleic acid of interest, liposome mediated transfection, and the like. For certain uses, it is preferred to target the nucleic acid to particular cells. In such instances, a vehicle used for delivering a nucleic acid according to the invention into a cell (e.g., a retrovirus, or other virus; a liposome) can have a targeting molecule attached thereto. For example, a molecule such as an antibody specific for a surface membrane protein on the target cell or a ligand for a receptor on the target cell can be bound to or incorporated within the nucleic acid delivery vehicle. For example, where liposomes are employed to deliver the nucleic acids of the invention, proteins which bind to a surface membrane protein associated with endocytosis may be incorporated into the liposome formulation for targeting and/or to facilitate uptake. Such proteins include proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half life, and the like. Polymeric delivery systems also have been used successfully to deliver nucleic acids into cells, as is known by those skilled in the art. Such systems even permit oral delivery of nucleic acids.

In the present invention, the preferred method of introducing exogenous genetic material into hematopoietic cells is by transducing the cells *in situ* on the matrix using replication-deficient retroviruses. Replication-deficient retroviruses are capable of directing synthesis of all virion proteins, but are incapable of making infectious particles. Accordingly, these genetically altered retroviral vectors have general utility for high-efficiency transduction of genes in cultured cells, and specific utility for use in the method of the present invention. Retroviruses have been used extensively for transferring genetic material into cells. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral

particles from tissue culture media, and infection of the target cells with the viral particles) are provided in the art.

The major advantage of using retroviruses is that the viruses insert efficiently a single copy of the gene encoding the therapeutic agent into the host cell genome, thereby permitting the exogenous genetic material to be passed on to the progeny of the cell when it divides. In addition, gene promoter sequences in the LTR region have been reported to enhance expression of an inserted coding sequence in a variety of cell types. The major disadvantages of using a retrovirus expression vector are (1) insertional mutagenesis, i.e., the insertion of the therapeutic gene into an undesirable position in the target cell genome which, for example, leads to unregulated cell growth and (2) the need for target cell proliferation in order for the therapeutic gene carried by the vector to be integrated into the target genome. Despite these apparent limitations, delivery of a therapeutically effective amount of a therapeutic agent via a retrovirus can be efficacious if the efficiency of transduction is high and/or the number of target cells available for transduction is high.

Yet another viral candidate useful as an expression vector for transformation of hematopoietic cells is the adenovirus, a double-stranded DNA virus. Like the retrovirus, the adenovirus genome is adaptable for use as an expression vector for gene transduction, i.e., by removing the genetic information that controls production of the virus itself. Because the adenovirus functions usually in an extrachromosomal fashion, the recombinant adenovirus does not have the theoretical problem of insertional mutagenesis. On the other hand, adenoviral transformation of a target hematopoietic cell may not result in stable transduction. However, more recently it has been reported that certain adenoviral sequences confer intrachromosomal integration specificity to carrier sequences, and thus result in a stable transduction of the exogenous genetic material.

Thus, as will be apparent to one of ordinary skill in the art, a variety of suitable vectors are available for transferring exogenous genetic material into hematopoietic cells. The selection of an appropriate vector to deliver a therapeutic agent for a particular condition amenable to gene replacement therapy and the optimization of the conditions for insertion of the selected expression vector into the cell, are within the scope of one of ordinary skill in the art without the need for undue experimentation. The promoter characteristically has a specific nucleotide sequence necessary to initiate transcription. Optionally, the exogenous genetic material further includes additional sequences (i.e., enhancers) required to obtain the desired gene transcription activity. For the purpose of this discussion an "enhancer" is simply any

nontranslated DNA sequence which works contiguous with the coding sequence (in cis) to change the basal transcription level dictated by the promoter. Preferably, the exogenous genetic material is introduced into the hematopoietic cell genome immediately downstream from the promoter so that the promoter and coding sequence are operatively linked so as to permit transcription of the coding sequence. A preferred retroviral expression vector includes an exogenous promoter element to control transcription of the inserted exogenous gene. Such exogenous promoters include both constitutive and inducible promoters.

Naturally-occurring constitutive promoters control the expression of essential cell functions. As a result, a gene under the control of a constitutive promoter is expressed under all conditions of cell growth. Exemplary constitutive promoters include the promoters for the following genes which encode certain constitutive or "housekeeping" functions: hypoxanthine phosphoribosyl transferase (HPRT), dihydrofolate reductase (DHFR) (Scharfmann et al., Proc. Natl. Acad. Sci. USA 88:4626-4630 (1991)), adenosine deaminase, phosphoglycerol kinase (PGK), pyruvate kinase, phosphoglycerol mutase, the actin promoter (Lai et al., Proc. Natl. Acad. Sci. USA 86: 10006-10010 (1989)), and other constitutive promoters known to those of skill in the art. In addition, many viral promoters function constitutively in eukaryotic cells. These include: the early and late promoters of SV40; the long terminal repeats (LTRS) of Moloney Leukemia Virus and other retroviruses; and the thymidine kinase promoter of Herpes Simplex Virus, among many others. Accordingly, any of the above-referenced constitutive promoters can be used to control transcription of a heterologous gene insert.

Genes that are under the control of inducible promoters are expressed only or to a greater degree, in the presence of an inducing agent, (e.g., transcription under control of the metallothionein promoter is greatly increased in presence of certain metal ions). Inducible promoters include responsive elements (REs) which stimulate transcription when their inducing factors are bound. For example, there are REs for serum factors, steroid hormones, retinoic acid and cyclic AMP. Promoters containing a particular RE can be chosen in order to obtain an inducible response and in some cases, the RE itself may be attached to a different promoter, thereby conferring inducibility to the recombinant gene. Thus, by selecting the appropriate promoter (constitutive versus inducible; strong versus weak), it is possible to control both the existence and level of expression of a therapeutic agent in the genetically modified hematopoietic cell. Selection and optimization of these factors for delivery of a therapeutically effective dose of a particular therapeutic agent is deemed to be within the

scope of one of ordinary skill in the art without undue experimentation, taking into account the above-disclosed factors and the clinical profile of the patient.

In addition to at least one promoter and at least one heterologous nucleic acid encoding the therapeutic agent, the expression vector preferably includes a selection gene, for example, a neomycin resistance gene, for facilitating selection of hematopoietic cells that have been transfected or transduced with the expression vector. Alternatively, the hematopoietic cells are transfected with two or more expression vectors, at least one vector containing the gene(s) encoding the therapeutic agent(s), the other vector containing a selection gene. The selection of a suitable promoter, enhancer, selection gene and/or signal sequence (described below) is deemed to be within the scope of one of ordinary skill in the art without undue experimentation.

The selection and optimization of a particular expression vector for expressing a specific gene product in an isolated hematopoietic cell is accomplished by obtaining the gene, preferably with one or more appropriate control regions (e.g., promoter, insertion sequence); preparing a vector construct comprising the vector into which is inserted the gene; transfecting or transducing cultured hematopoietic cells *in vitro* with the vector construct; and determining whether the gene product is present in the cultured cells.

Table 1. Human Gene Therapy Protocols Approved by RAC: 1990-1994

Severe combined immune deficiency (SCID) due to ADA deficiency	Autologous lymphocytes transduced with human ADA gene	7/31/90
Advanced cancer	Tumor-infiltrating lymphocytes transduced with tumor necrosis factor gene	7/31/90
Advanced cancer	Immunization with autologous cancer cells transduced with tumor necrosis factor gene	10/07/91
Advanced cancer	Immunization with autologous cancer cells transduced with interleukin-2 gene	10/07/91
Asymptomatic patients infected with HIV-1	Murine Retro viral vector encoding HIV-1 genes [HIV-IT(V)]	6/07/93
AIDS	Effects of a transdominant form of <i>rev</i> gene on AIDS intervention	6/07/93
Advanced cancer	Human multiple-drug resistance (MDR) gene transfer	6/08/93
HIV infection	Autologous lymphocytes transduced with catalytic ribozyme that cleaves HIV-1 RNA (Phase I study)	9/10/93
Metastatic melanoma	Genetically engineered autologous tumor vaccines producing interleukin-2	9/10/93
HIV infection	Murine Retro viral vector encoding HIV-IT(V) genes (open label Phase I/II trial)	12/03/93
HIV infection (identical twins)	Adoptive transfer of syngeneic cytotoxic T lymphocytes (Phase I/II pilot study)	3/03/94

Breast cancer (chemo-protection during therapy)	Use of modified Retro virus to introduce chemotherapy resistance sequences into normal hematopoietic cells (pilot study)	6/09/94
Fanconi's anemia	Retro viral mediated gene transfer of the Fanconi anemia complementation group C gene to hematopoietic progenitors	6/09/94
Metastatic prostate carcinoma	Autologous human granulocyte macrophage-colony stimulating factor gene transduced prostate cancer vaccine *(first protocol to be approved under the accelerated review process; ORDA=Office of Recombinate DNA Activities)	ORDA/NIH 8/03/94*
Metastatic breast cancer	<i>In vivo</i> infection with breast-targeted Retro viral vector expressing antisense <i>c-fos</i> or antisense <i>c-myc</i> RNA	9/12/94
Metastatic breast cancer (refractory or recurrent)	Non-viral system (liposome-based) for delivering human interleukin-2 gene into autologous tumor cells (pilot study)	9/12/94
Mild Hunter syndrome	Retro viral-mediated transfer of the iduronate-2-sulfatase gene into lymphocytes	9/13/94
Advanced mesothelioma	Use of recombinant adenovirus (Phase I study)	9/13/94

The foregoing (Table 1), represent only examples of genes that can be delivered according to the methods of the invention. Suitable promoters, enhancers, vectors, etc., for such genes are published in the literature associated with the foregoing trials. In general, useful genes replace or supplement function, including genes encoding missing enzymes such as adenosine deaminase (ADA) which has been used in clinical trials to treat ADA deficiency and cofactors such as insulin and coagulation factor VIII. Genes which affect regulation can also be administered, alone or in combination with a gene supplementing or replacing a specific function. For example, a gene encoding a protein which suppresses expression of a particular protein-encoding gene can be administered. The invention is particularly useful in delivering genes which stimulate the immune response, including genes encoding viral antigens, tumor antigens, cytokines (e.g. tumor necrosis factor) and inducers of cytokines (e.g. endotoxin).

Employing the culture conditions described in greater detail below, it is possible according to the invention to preserve hematopoietic progenitor cells and to stimulate the expansion of hematopoietic progenitor cell number and/or colony forming unit potential. Once expanded, the cells, for example, can be returned to the body to supplement, replenish, etc. a patient's hematopoietic progenitor cell population. This might be appropriate, for example, after an individual has undergone chemotherapy. There are certain genetic conditions wherein hematopoietic progenitor cell numbers are decreased, and the methods of the invention may be used in these situations as well.

It also is possible to take the increased numbers of hematopoietic progenitor cells produced according to the invention and stimulate them with hematopoietic growth agents that promote hematopoietic cell maintenance, expansion and/or differentiation, and also influence cell localization, to yield the more mature blood cells, *in vitro*. Such expanded
5 populations of blood cells may be applied *in vivo* as described above, or may be used experimentally as will be recognized by those of ordinary skill in the art. Such differentiated cells include those described above, as well as T cells, plasma cells, erythrocytes, megakaryocytes, basophils, polymorphonuclear leukocytes, monocytes, macrophages, eosinophils and platelets.

10 In all of the culturing methods according to the invention, except as otherwise provided, the media used is that which is conventional for culturing cells. Examples include RPMI, DMEM, Iscove's, etc. Typically these media are supplemented with human or animal plasma or serum. Such plasma or serum can contain small amounts of hematopoietic growth factors. The media used according to the present invention, however, can depart from that
15 used conventionally in the prior art. In particular, it has been discovered, surprisingly, that hematopoietic progenitor cells can be cultured on the matrices described above for extended periods of time without the need for adding any exogenous growth agents (other than those which may be contained in plasma or serum, hereinafter "serum"), without inoculating the environment of the culture with stromal cells and without using stromal cell conditioned
20 media. Prior to the present invention, at least one of the foregoing agents was believed necessary in order to culture hematopoietic progenitor cells.

The growth agents of particular interest in connection with the present invention are hematopoietic growth factors. By hematopoietic growth factors, it is meant factors that influence the survival, proliferation or differentiation of hematopoietic progenitor cells.
25 Growth agents that affect only survival and proliferation, but are not believed to promote differentiation, include the interleukins 3, 6 and 11, stem cell factor and FLT-3 ligand. Hematopoietic growth factors that promote differentiation include the colony stimulating factors such as GM-CSF, G-CSF, M-CSF, Tpo, Epo, Oncostatin M, and interleukins other than IL-3, 6 and 11. The foregoing factors are well known to those of ordinary skill in the art.
30 Most are commercially available. They can be obtained by purification, by recombinant methodologies or can be derived or synthesized synthetically.

"Stromal cell conditioned medium" refers to medium in which the aforementioned lymphoreticular stromal cells have been incubated. The incubation is performed for a period

sufficient to allow the stromal cells to secrete factors into the medium. Such "stromal cell conditioned medium" can then be used to supplement the culture of hematopoietic progenitor cells promoting their proliferation and/or differentiation.

Thus, when cells are cultured without any of the foregoing agents, it is meant herein
5 that the cells are cultured without the addition of such agent except as may be present in serum, ordinary nutritive media or within the blood product isolate, unfractionated or fractionated, which contains the hematopoietic progenitor cells.

According to another aspect of the invention a method for *in vivo* maintenance,
expansion and/or differentiation of hematopoietic progenitor cells is provided. The method
10 involves implanting into a subject a porous solid matrix having seeded hematopoietic progenitor cells, hematopoietic progenitor cell progeny, and lymphoreticular stromal cells. Implantation of matrices similar to the matrices of the invention is well known in the art (Stackpool, GJ, et al, Combined Orthopaedic Research Societies Meeting, Nov. 6-8, 1995, San Diego, CA, Abstract Book p. 45; Turner, TM, et al., 21st Annual Meeting of the Society
15 for Biomaterials, March 18-22, San Francisco, CA, Abstract Book p. 125). Such matrices are biocompatible (i.e., no immune reactivity-no rejection) and can be implanted and transplanted in a number of different tissues of a subject. Such methods are useful in a variety of ways, including the study of hematopoietic progenitor cell maintenance, expansion, differentiation and/or localization *in vivo*, in a number of different tissues of a subject, and/or between
20 different subjects.

As used herein, a subject is a human, non-human primate, cow, horse, pig, sheep, goat, dog, cat or rodent. Human hematopoietic progenitor cells and human subjects are particularly important embodiments. As described above, when the matrices of the invention are used for such *in vivo* implantation studies, biological agents that promote angiogenesis
25 (vascularization) and/or prevent/reduce inflammation may also be used for coating of the matrices. Preferred biological agents are as described above. Also as described above, the hematopoietic progenitor cells are pre-seeded onto the porous solid matrix and cultured *in vitro* according to the invention, before implantation into a subject. According to the invention, an amount of the cells is introduced *in vitro* into the porous solid matrix, and co-
30 cultured with lymphoreticular stromal cells in an environment that is free stromal cell conditioned medium, and exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion and/or differentiation, other than serum. Implantation is then carried out. In certain embodiments, stromal cell conditioned medium

and exogenous hematopoietic growth factors may be added during the *in vitro* culture before implantation.

According to one aspect of the invention, a method for inducing T cell reactivity/activation, *in vitro*, is provided. Induction of T cell reactivity/activation involves co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells in the presence of antigen presenting cells and an antigen, in one of the foregoing matrices, under conditions sufficient to induce the formation of T cells and/or T cell progenitors from the hematopoietic progenitor cells having specificity for the antigen. The foregoing conditions could easily be established by a person of ordinary skill in the art, without undue experimentation (see also Sprent J, et al., *J Immunother*, 1998, 21(3):181-187; Berridge MJ, *Crit Rev Immunol*, 1997, 17(2):155-178; Owen MJ, et al., *Curr Opin Immunol*, 1996, 8(2):191-198; Whitfield JF, et al., *Mol Cell Biochem*, 1979, 27(3):155-179; Fauci AS, et al., *Ann Intern Med*, 1983, 99(1):61-75). Antigen stimulation of T cells in the presence of APCs, induces an antigen specific response that can be measured using a proliferation assay or just by measuring IL-2 production (see discussion below). These cells can be detected by culturing T cells with antigen at an appropriate concentration (e.g., 0.1-1.0 μ M tetanus toxoid) in the presence of APCs. If antigen specific T cells are present they can be detected using the assays described below under self-tolerance/anergy. Stimulation of T cells in the presence of APCs may include co-stimulation with a co-stimulatory agent. Co-stimulatory agents include lymphocyte function associated antigen- 3 (LFA-3), CD2, CD40, CD80/B7-1, CD86/B7-2, OX-2, CD70, and CD82. Co-stimulatory agents may also be used in lieu of APCs, provided that MHC class II molecules and anti-CD3 antibodies are co-administered with the co-stimulatory agent(s).

One or more antigens can be used at the same time for incubation in the foregoing culture system. Preferably, the lymphoreticular stromal cells are thymic stromal cells and of murine origin when the hematopoietic progenitor cells being expanded are human. Therefore, large numbers of antigen-specific mature T and immature T cells may be obtained in a short period of time that were never before realized using existing art methodologies. The present invention thus becomes useful in a wide range of applications, including pre-exposure vaccination of individuals with *in vitro* primed T cells, treatment of cancer patients using tumor-targeted T cell immunotherapy, treatment of bone marrow transplant patients (for whom opportunistic infections, such as CMV, are problematic and yet amenable to treatment with targeted T cells such as CMV-targeted cytotoxic lymphocytes), enhancement of

conventional vaccination efficacy through T cell adjuvant therapy, treatment of outbreaks of emergent or re-emergent pathogens, etc. The antigen presenting cells include cells such as dendritic cells, monocytes/macrophages, Langerhans cells, Kupfer cells, microglia, alveolar macrophages and B cells, and methods for their isolation are well known in the art. The antigen presenting cells may also be derived from hematopoietic progenitor cells *in vitro*.

Immunological tolerance refers to the inhibition of a subject's ability to mount an immune response, e.g., to a donor antigen, which would otherwise occur in response to the introduction of a non-self antigen into the subject. Tolerance can involve humoral, cellular, or both humoral and cellular responses. Thymic education results in the generation of T cells capable of responding to a myriad of foreign antigens in the context of *self*-MHC, but not *self*-antigens alone. This is achieved primarily by a systematic rescue of appropriate thymocytes from programmed cell death, based on a theme of *self*-restriction, and the release of these cells into the periphery to serve as self-tolerant T cells.

Self-tolerance can be established *in vitro* under conditions known in the art that include coculturing CD34⁺ T progenitors derived from a donor (A), in the presence of thymic stroma from another individual (B). Briefly, thymic stroma is established from freshly isolated thymic tissue that is digested into a single cell suspension using a collagenase (20 µg/ml, Sigma Chemical Co.). Thymic stromal cultures are established by plating the cell suspension in 24 well plates at a concentration of 4 x 10⁶ viable cells per well in a volume of 2 ml R10 (RPMI plus 10%FCS). Cultures are incubated in a standard humidified tissue culture incubator at 37° C with 5% CO₂. After one to two days, non-adherent cells are removed by washing three times with R10. The stroma requires an additional 7-10 days to become confluent. The stroma is maintained in R10 which is changed at least twice per week. After 7-10 days in culture, CD34⁺ cells in R10 are added to the stroma at a concentration of 1-3 x 10⁵ cells per well. Cultures are fed bi-weekly using partial medium exchanges with R10 with no exogenous cytokines added to these cultures. After 14-21 days, the non-adherent cells are removed from the cultures. The remaining, attached cells are self-tolerant T cells that have developed *in vitro*.

Methods for determining if tolerance has been established *in vitro* are also known to a person of ordinary skill in the art, and involve measurement of a proliferative response to: *self* (A), as well as to the thymus donor (B), and a third party (C), peripheral blood mononuclear

cells (PBMCs). Briefly, PBMCs from A, B and C are prepared by Ficoll gradient centrifugation.

1 x 10⁵ responder cells (*in vitro* generated T cells from A) are plated out in multiple replicates in a 96 well plate. Stimulator cells (PBMCs from A, B and C) are irradiated (3000 Rads) and added in 12 replicates at 1 x 10⁵ cells per well. Con-A (5 µg/ml) is used as a positive control. After 4 days 1 µCi of ³H-Thymidine is added to each well, and the plates harvested 18-24 hours later. If tolerance has been established, the *in vitro* generated T cells will respond and proliferate when mixed with an unrelated third party (C), but do not proliferate when mixed with PBMCs from self (A) or the thymic donor (B).

According to another aspect of the invention, a method for inducing T cell anergy, *in vitro*, is provided. Induction of T cell anergy involves co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells in one of the foregoing matrices, in the presence of antigen under conditions sufficient to induce the formation of T cells and/or T cell progenitors and to inhibit activation of the formed T cells and/or T cell progenitors.

Anergy is defined as an unresponsive state of T cells (that is they fail to produce IL-2 on restimulation, or proliferate when restimulated)(Zamoyska R, *Curr Opin Immunol*, 1998, 10(1):82-87; Van Parijs L, et al., *Science*, 1998, 280(5361):243-248; Schwartz RH, *Curr Opin Immunol*, 1997, 9(3):351-357; Immunol Rev, 1993, 133:151-76). Anergy may, however, be irreversible. Anergy may be induced via antigen-specific T cell stimulation in the absence of co-stimulation (one signal vs. two signal hypothesis). Alternatively peptides of low affinity or very high concentrations of peptide even in the presence of co-stimulation can induce anergy. Anergy can be induced *in vitro* by culturing T cells in the absence of antigen presenting cells (B cells, macrophages or dendritic cells). These T cells are then exposed to antigen for example tetanus toxoid (e.g., 0.1-1.0 µM). An aliquot of the T cells is used to present antigen. This constitutes antigen presentation without co-stimulation and will induce anergy (Nelson A, et al., *In Immuno*, 1998, 10(9):1335-46). Alternatively T cells can be cocultured with APCs, in the context of very high (10-100 µM) or very low (0.01-0.05 µM) tetanus toxoid, which will induce a state of unresponsiveness.

Anergy can be measured by taking the T cells described above, and restimulating them with antigen (e.g., 0.1-1.0 µM tetanus toxoid) in the presence of APCs. If the cells are anergic they will not respond to antigen at an appropriate concentration in the context of APCs. Anergy is measured by culturing the cells as such for 3-5 days and measuring proliferation or

the lack thereof as follows. Briefly APCs are plated out in multiple replicates in a 96 well plate, after irradiation (3000 Rads). These cells are pulsed with antigen (e.g., 0.1-1.0 μ M) for 2 hours, and then T cells are added in 12 replicates at 1×10^5 /cells per well. Con-A (5 μ g/ml) is used as a positive control. After 4 days 1 μ Ci of 3 H-Thymidine is added to each well, and the cells are harvested 18-24 hours later. If the cells are anergic they will not proliferate in response to antigen stimulation. Alternatively, the production of IL-2 can be measured in the supernatants of the cultures described above. Supernatants are collected daily and IL-2 production is measured using a commercial ELISA assay. An additional approach includes flow cytometry based staining specific for intracellular expression of the cytokines IL-2, γ IFN and TNF α using antibodies specific to the human forms of these factors (Becton Dickinson). Further, semiquantitative RT-PCR of mRNA for these factors can also be used.

According to another aspect of the invention, a method for identifying an agent suspected of affecting hematopoietic cell development is provided. The method involves introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix of the invention, and co-culturing in a test co-culture the hematopoietic progenitor cells and the lymphoreticular stromal cells in the presence of at least one candidate agent suspected of affecting hematopoietic cell development. By “hematopoietic cell development” it is meant to include hematopoietic progenitor cell maintenance, expansion, differentiation, and/or cell-death apoptosis (programmed cell-death). “Maintenance” includes the hematopoietic progenitor cell’s ability to maintain its pluripotentiality. “Expansion” includes the hematopoietic progenitor cell’s ability to divide and grow, and “differentiation” includes the hematopoietic progenitor cell’s ability to differentiate toward a specific cell lineage. “Cell-death” also includes programmed cell-death (apoptosis). By “affecting” hematopoietic cell development it is therefore meant to include effects on hematopoietic progenitor cell maintenance, expansion, differentiation, and/or cell-death. Such effect (or influence) can be either positive or negative/inhibitory in nature. For example, a positive effect would be maintenance of pluripotentiality of the progenitor cells, and/or increase in the number of the pluripotential progenitor cells. A negative effect would lead into the differentiation of the progenitor cells and loss of pluripotentiality, or even progenitor cell-death. A negative effect on a particular cell population may also have a positive effect on a different cell population. For example, an inhibitory effect on a B cell lineage may result in a positive effect on, for example, a T cell lineage. The agent suspected of affecting hematopoietic cell development may be administered in the form of a transfected

nucleic acid into the lymphoreticular stromal cells as well as being added straight into the media.

To determine whether the at least one candidate agent affects hematopoietic cell development in the test co-culture, the phenotype and/or genotype (as well as the numbers) of the hematopoietic cells generated in the test co-culture is compared to the phenotype and/or genotype (and numbers) of hematopoietic cells generated in a control co-culture. The control co-culture is performed under identical conditions to the test co-culture (i.e., identical initial numbers and types of both hematopoietic progenitor cells and lymphoreticular stromal cells, in an identical matrix, identical culture media, etc.), but with the exception that the at least one candidate agent suspected of affecting cell hematopoietic cell development is omitted from the control co-culture. Methods for determining the phenotype and/or genotype of hematopoietic cells are well known in the art, and a few examples can be found throughout this application.

In yet another aspect of the invention, a method for isolating from a cell culture an agent suspected of affecting hematopoietic cell development is also provided. The method involves introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix of the invention, co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells and obtaining a test-supernatant (or a fraction thereof) from the co-culture. The test-supernatant (or a fraction thereof) is then compared to a control-supernatant (or a fraction thereof). By "comparing" it is meant that a profile of agents (suspected of affecting hematopoietic cell development) present in the test-supernatant and secreted from the cells of the co-culture, is compared to a similar profile of agents present in the control-supernatant and secreted from the cells of a control culture or co-culture. Methods of obtaining such profiles of secreted agents are well known in the art and include two-dimensional (2-D) gel electrophoresis. Other methods also include various types of HPLC, thin layer chromatography.

A "control culture or co-culture" may involve the culture of hematopoietic progenitor cells in a parallel culture system known in the art (e.g. U.S. Patent No. 5,677,139 by Johnson *et al.*), in order to obtain a result that correlates (i.e. approximates) to the result established in the co-culture system of the invention. For example, a test co-culture according to the invention that involves the co-culture of human hematopoietic progenitor cells and lymphoreticular stromal cells from a mouse thymus, gives rise to a diverse (a variety of sub-types) population

of human lymphoid cells committed to the T cell lineage. The test-supernatant obtained from such co-culture is then compared to a control-supernatant obtained from a culture of human hematopoietic progenitor cells in a parallel system of the prior art (as described above) that also gives rise to a population of human lymphoid cells committed to the T cell lineage.

5 Other examples of control cultures or co-cultures may include the co-culture of hematopoietic progenitor cells with lymphoreticular stromal cells of different tissue origin to the ones used in the test co-culture in the matrix of the invention. Additionally, the tissue may or may not be of lymphoid origin. A person of ordinary skill in the art would be able to easily choose and establish such control cultures or co-cultures. Once the profiles of agents suspected of

10 affecting hematopoietic cell development are obtained, a subfraction of the test-supernatant that contains an agent suspected of affecting hematopoietic cell development that appears to be different or absent from the control-supernatant, can then be isolated and further characterized. For example, a candidate agent that appears to be migrating differently in a 2-D gel electrophoresis blot of the test-supernatant can be purified and further characterized

15 using methods such as protein sequencing and mass spectrometry. Agents that appear in the 2-D gel electrophoresis blot but are absent from the blot of the test-supernatant are also suspect of affecting hematopoietic cell development and can be further purified.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention

20 and are not to be construed to limit the scope of the invention.

Examples

Experimental Procedures

Isolation of Human CD34⁺ cells

Five to ten milliliters of venous umbilical cord blood (UCB) was extracted using a

25 heparinized syringe prior to the severing of the umbilical cord during a Caeserean section delivery of a human embryo. After the umbilical cord was severed and the infant delivered, the placenta was removed by clamping the umbilical vein proximally and severing distally to the placenta. Immediately after the placenta was removed the umbilical vein was unclamped and the blood contained in the placenta drained into an appropriate heparinized container.

30 Before processing, the cord and placenta blood was mixed together. After extraction the cord/placenta blood was diluted 2:1 with washing media (RPMI 1640, 10 IU/ml penicillin, 10 µg/ml streptomycin, 1 mM L-glutamine). The sample(s) were then underlayered with a volume of Ficoll-Hypaque (1.077 g/ml) equal to half of the diluted sample volume so that a distinct

sample/Ficoll interface formed. After centrifugation for 45 minutes at 400 x g the interface containing mononuclear cells was removed. The cells were then washed by resuspending in culture medium and centrifuging for 10 minutes at 400 x g. The resulting pellet was resuspended in 6 ml of ammonium chloride lysing buffer (0.15M NH₄Cl, 1.0 mM KHCO₃, 5 0.1M Na₂EDTA) for 3 minutes to lyse any remaining erythrocytes. The suspension was then diluted with media and washed twice more. After the final wash the cells were resuspended in 1-2 ml media and the number of viable cells was determined by trypan blue exclusion.

Human CD34⁺ progenitor cells were also prepared from disaggregated human fetal thymus obtained from 16-22 week old abortuses. For disaggregation procedures see below 10 under *Mouse Thymic Stroma*.

Cells expressing the surface antigen CD34 were isolated using the Dynal CD34 Progenitor Cell Selection System (Dynal, Lake Success, N.Y.) or the MiniMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). The mononuclear cells isolated from UCB (or bone marrow) were suspended in isolation buffer (PBS, 2% heat inactivated fetal bovine 15 serum, 10 IU/ml penicillin, 10 µg/ml streptomycin) at a concentration of 2.5×10^7 cells/ml. The suspension was then added to magnetic anti-human CD34 beads (Dynal M-450 CD34) in a ratio of 4.0×10^7 beads per ml of suspension, in a round bottom tube. (Dynabeads M-450 CD34 are superparamagnetic beads bound to monoclonal antibody specific for CD34). The mixture was vortexed gently and incubated at 4° C for 45 minutes with gentle tilt rotation 20 using a Dynal Sample Mixer. After incubation the bead/cell mixture was resuspended in a larger volume of isolation buffer and placed in a magnetic separation device for 2 minutes to allow the cell/bead complexes to accumulate to the tube wall. While still exposed to the agent, the suspension containing the cells not bound to the magnetic beads was aspirated. The cell/bead complexes were washed three more times in this manner, pooling the suspensions 25 containing the CD34 negative cells into the same tube. The tube containing the released cells (CD34-) was then placed on the magnetic separator to remove any remaining beads and this supernatant was transferred to a new conical tube. All CD34⁺ cells attached to beads were washed twice in a minimum of 10 ml of isolation buffer with centrifugation at 2000 rpm for 8 min. Cells bound to magnetic beads were then resuspended in 100 µl of isolation buffer per 30 4×10^7 beads used, with a minimum volume of 100 µl. The CD34 positive cells were then detached from the beads by adding an equal volume of an anti-idiotypic antibody (DETACHaBEAD CD34, Dynal), vortexing, and gently mixing at room temperature using a

Dynal Sample Mixer for one hour. The cells were isolated from the cell/bead suspension by adding isolation buffer and placing the tube in the magnetic separation device for 2 minutes. After the beads migrated to the tube wall, the supernatant containing the CD34 positive cells was transferred to a new tube. The beads were washed three more times with the suspensions
5 containing the released cells pooled into the same tube. The tube containing the released CD34+ cells was then placed on the magnetic separator to remove any remaining beads, and the supernatant was transferred to a new conical tube. The cells were washed twice in a minimum of 10 ml of isolation buffer with centrifugation at 2000 rpm for 10 minutes.

Alternatively, human bone marrow was obtained by posterior iliac crest aspiration
10 from healthy adult volunteers in accordance with institutional review board guidelines and after giving informed consent. 10-15 mL of human bone marrow was collected in an heparinized sterile syringe, transported at room temperature and used within 6 hours. Bone marrow was diluted in a 5-times volume of PBS and the mononuclear cells (MNCs) separated by density gradient centrifugation over a column of Ficoll-Paque (Pharmacia Biotech Inc.,
15 Piscataway, NJ). MNCs thus obtained were washed twice in 10 mL PBS, and the remaining erythrocytes removed by lysis with ACK Lysing Buffer (Bio Whittaker, Walkersville, MD).

In order to select a more immature phenotype of progenitor cell within the CD34⁺ population, we elected to use an immunomagnetic bead selection system employing an antibody to the novel stem cell antigen, AC133. AC133 is a 5-transmembrane cell surface
20 antigen expressed on 20-60% of human CD34⁺ cells, including the CD38^{neg/dim} subset (representing the non-lineage-committed precursors) but is not expressed on mature leukocytes (Yin AH, et al., *Blood*, 1997, 90:5002-12; Nfiraglia S, et al., *Blood*, 1997, 90: 5013-21; Buhning HJ, et al., *Ann N Y Acad Sci*, 1999, 872: 25, discussion 38-9). Although a small number of mature CD2⁺ T-cells were transferred into our co-cultures with the AC133⁺
25 progenitors we do not believe that the T-cells generated in this system are derived from either CD2⁺ mature lymphocytes or CD2⁺ lymphoid-committed precursors. We, and others (Fisher AG, et al., *Int Immunol*, 1990, 2:571-8), have observed that the deliberate introduction of mature human T-cells into the co-cultures does not result in increased numbers of T-cells or their precursors. The AC133⁺ MNC fraction was isolated by immunomagnetic bead selection
30 using an AC133 Cell Isolation Kit (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer's protocols.

Mouse Thymic Stroma:

Thymi were obtained from freshly sacrificed 6 week-old B6 (BALB/C) mice. Thymi were physically disaggregated with surgical scissors in order to produce a cell suspension which also contained fragments of thymic tissue less than 0.5mm³ in size. The cell suspension containing thymic fragments was plated onto 0.5cm x 0.5cm x 0.2cm pieces of Cellfoam (80 ppi), placed in each well of a 24 well plate. Each well contained at least 5 x 10⁶ cells and 4 fragments of fetal thymus per Cellfoam block and the cells were cultured in fully supplemented IMDM. The medium in thymic cultures was changed initially at 48 hours post establishment of the culture and at three day intervals there after. On average 80% confluent thymic stromal monolayers were established on Cellfoam between 10 and 14 days. At 10 to 14 days of culture the Cellfoam blocks each containing a sub-confluent layer of thymic stroma were removed from the 24 well plate and placed in the wells of a new 24 well plate, and co-cultured with CD34⁺ cells.

Human CD34⁺/Murine Thymic Stroma Co-Culture conditions:

Five thousand CD34⁺ cells derived from UCB or human bone marrow were then plated onto the irradiated murine thymic stroma. In the case where Cellfoam was used, CD34⁺ cells were plated directly onto the Cellfoam itself in the well of the 24 well tissue culture dish. Medium in co-cultures was changed every three days and was not supplemented with exogenous cytokines. Cells generated from the CD34⁺ cells were harvested at 7 days post establishment of the co-culture and flow-cytometric and functional studies were performed on the derived cells.

Assessment of Immunophenotype and Function of Cells Derived from the Co-cultures:

Adherent cells were harvested with a non-trypsin isolation solution (Cell Dissociation Solution, Sigma, St. Louis, MO) to minimize alteration of surface staining characteristics. To recover adherent cells from Cellfoam, units were washed twice by immersion into PBS, saturated by brief vortexing in an excess of Cell Dissociation Solution, incubated for 20 minutes at 37°C, and centrifuged at 1500 rpm for 10 minutes.

Cells were harvested by gentle aspiration and washed twice in PBS. Harvested cells were counted and assessed for viability by trypan blue exclusion. After counting, cells were stained in a final volume of 100µL with 2% mouse serum (Dako, Carpinteria, CA) and the following fluorochrome-conjugated antibodies: TCRαβ, TCRγδ, CD2, CD3, CD4, CD8, CD14, CD33 and CD34 (Becton Dickinson, San Jose, CA). Conjugated isotype control antibodies for all four fluorochromes (FITC, PE, Peridinin chlorophyll protein (PerCP), and

Allophycocyanin (APC) were used for each culture. Stained samples were washed three times with PBS, fixed with 1% paraformaldehyde, and analyzed with a FACScalibur flow cytometer (Becton Dickinson). Appropriate controls included matched isotype antibodies to establish positive and negative quadrants, as well as appropriate single color stains to establish compensation. For each sample, at least 10,000 list mode events were collected. Anti-CD3 and anti-CD14 were utilized to detect contaminating T-cells and monocytes in the CD34⁺ selected MC subpopulation.

Human leukocytes were distinguishable from murine cells on immunophenotypic analysis by gating on the CD45⁺ population. After 14 days in co-culture, >70% of CD45⁺ cells coexpressed CD3, CD4, and/or CD8. It was possible to track the sequential differentiation of T-lymphoid precursors in this system over 2 weeks (Figure 1). CD34⁺ progenitors added into co-culture with a murine thymic stroma cells, on a three-dimensional matrix (Cellfoam). Non-adherent cells were harvested 7, 14 and 21 days after establishment of the co-cultures and their immunophenotype determined by FACS analysis. The data in panel (a) demonstrate the acquisition of CD2 and the down-regulation of the hematopoietic progenitor cell marker, CD34. Acquisition of cell surface CD4 and CD8 markers occurred after 14 days in coculture; (b): discrete populations of SP CD4⁺ and SP CD8⁺ are demonstrated including their DP CD4⁺CD8⁺ precursors. Acquisition of CD4 at day 14 was associated with acquisition of CD3; (c and d): all CD4⁺ cells co-expressed CD3. CD3 was co-expressed with the majority of CD8⁺ cells; those cells which were CD3⁻CD8⁺ were found to express TCR $\gamma\delta$. TCR $\alpha\beta$ was expressed by 78% of CD3⁺ cells although a smaller population (20%) of CD3⁺ cells expressing TCR $\gamma\delta$ was also detectable (6% CD3⁺CD8⁺ TCR $\gamma\delta$, 14% CD3⁺CD8⁺ TCR $\gamma\delta$).

T cell Function:

T cell function was assessed by determining CD69 expression in response to mitogens and ³H-Thymidine uptake in response to the mitogen Con-A. T cells generated in the co-culture system were also examined for their infectability by HIV-1 and their transducability by the MFG murine retroviral vector. T cells generated from the co-culture showed expected high levels of ³H-Thymidine uptake (10 x control unresponsive cells) in response to the mitogen ConA and a four-fold increase in the expression of the activation marker CD69 as determined by flow cytometry.

HIV-1 Challenge of T-cells generated from HPC/thymic stromal co-cultures:

T-cells generated from HPCs were challenged with T-cell tropic isolate HIV_{IIIB} at a multiplicity of infection of 1. Titered stocks of HIV-1 were generated by standard means well known in the art. Samples of culture supernatant were removed from cultures at 3, 6, 9, 14 and 28 days post HIV challenge for HIV-1 p24 antigen estimation by ELISA (Coulter, Miami, FL). Secondly, sorted CD4⁺ T cells generated from co-cultures of BM HPCs with thymic stroma were challenged with HIV_{IIIB} at a multiplicity of infection of 1. Cell viability was also determined following challenge of monocytes and T cells with HIV-1 using trypan blue exclusion. T cells generated from HPCs were infectable with HIV-1 and produced up to 0.69ng/ml of HIV-1 p24 by day 10 of culture. Both unsorted and sorted T cells generated from the co-cultures of HPCs on murine thymic stroma on Cellfoam and exposed to heat inactivated HIV_{IIIB}, produced undetectable levels of HIV-1 p24. The viability of T cells also declined significantly following exposure to infections HIV-1. The levels of HIV-1 p24 antigen production in T cells generated from the Cellfoam co-culture system was similar to levels of HIV-1 p24 production from human activated peripheral blood T cells.

Transduction of T cells generated from HPC/thymic stromal co-cultures with an amphotropic murine retroviral vector:

T cells generated from HPCs and expanded in IL-2 and PHA were exposed to the murine retrovirus based vector, MFG, encoding the intranuclear localized enzyme β -galactosidase at an M.O.I. of 10 on three occasions over the period of 72 hours. Titered retroviral vector was generated by standard means from a human based FLYA4 packaging cell-line. T cells were also exposed to heat inactivated MFG. Transduced cells were harvested from cultures at 7 days following retroviral exposure and stained by standard methods for the expression of the beta-galactosidase transgene. Transduction efficiencies of between 12 and 26% were observed in T cells generated from co-cultures of HPCs with murine thymic stroma grown on Cellfoam. No β -galactosidase activity was detectable in T cells exposed to the heat inactivated retroviral vector. The transduction efficiency of human T cells generated from the Cellfoam co-culture system is similar to that seen in activated peripheral blood T cells.

mRNA extraction and cDNA synthesis:

Generated cells were also lysed and RNA was prepared from the cells for RNA PCR in order to determine T cell receptor gene expression. Messenger RNA was extracted from cells grown on a thymic monolayer. The extraction was performed using guanidinium thiocyanate and oligo-dT spun columns (QuickPrep Micro mRNA Purification Kit;

Pharmacia, Piscataway, N.J.) according to the manufacturer's instructions. mRNA samples were stored at -70° C. The first strand cDNA was synthesized in a 40 µl final volume, using approximately 2 µg of mRNA, 1 µg of random primer, and 6.25 units of AMV reverse transcriptase (GIBCO/BRL). Samples were incubated for 10 minutes at room temperature, 1 hour at 42° C., 5 minutes at 95° C., and 5 minutes at 4° C. RT-PCR for a number of lymphoid-specific genes (including RAG-2) was performed using reverse transcription using random primers and Moloney MuLV reverse transcriptase (GIBCO-BRL, Grand Island, NY). cDNAs were amplified using gene-specific primers, e.g., for the human RAG-2 gene which is expressed transiently only by cells undergoing lymphocyte differentiation, Vβ gene expression, and the like. PCR amplification were performed in a GeneAmp 9600 thermal cycler (Perkin Elmer, Norwalk, CT) using conditions well known in the art.

Example 1: *Viability, Immunophenotype and Function of Human Cells Generated In Co-Culture Systems*

The numbers of viable cells generated in the co-culture system and their immunophenotype are shown in Table 2. Maximal human T cell proliferation was seen when human fetal thymic CD34+ cells and UCB CD34+ cells were co-cultured with murine fetal thymic stroma grown on Cellfoam. Data generated from a direct comparison of co-culture of CD34+ cells on murine thymic stroma on cell foam versus co-culture of CD34+ cells on murine stroma grown as a simple monolayer are also shown in Table 2.

T cells generated in the co-culture system were also shown to be infectable by T-tropic HIV-1_{IIIB} and these cells were also transducible at a transduction efficiency of 12-22% (n=3) with MFG vector.

Example 2: *Maintenance of Immature Progenitor Cells*

According to the invention, it has also been discovered that Cellfoam cultures of thymic stromal cells are able to induce T cell differentiation of CD34⁺ progenitors and yet preserve a fraction of CD34⁺ cells. Primate CD34⁺ progenitors were cultured on either human or swine thymus that had been established on Cellfoam tissue scaffolds. After 14-21 days, CD3+CD4+CD8+ triple positive cells and CD3+CD4+ and CD3+CD8+ double positive cells are reliably recovered. In addition, the CD3- cell fraction was found to contain CD34⁺ progenitor cells after 14-21 days. These CD34⁺ cells not only were CD3-, but many were also CD2+. This demonstrates that thymus cultures in Cellfoam tissue scaffolds can support T cell differentiation while simultaneously preserving the long-lived CD34⁺ progenitor cell population. As will be evident to those skilled in the art, this surprising finding indicates that

ongoing differentiation of T progeny while maintaining immature progenitor cells is possible in Cellfoam.

Example 3: T Cell Function (Proliferation/Anergy) Assays

T cell function is evaluated by the proliferative potential to specific and non-specific antigens using standard assays. Specifically, the assay assesses the response of T cell receptor (TCR) mediated proliferation using anti-CD3 antibodies (Becton Dickinson) as well as baseline non-specific proliferation using concavalin A (Con-A). Briefly, T cells are washed and resuspended in RPMI with 10% FCS at a concentration of 10^6 cells/ml. 100 μ l (10^5 cells) are added to each well of a 96 well plate. Cells are stimulated with either Con-A (5 μ g/ml) (non-specific response) or monoclonal antibodies to CD3 in the presence of IL-2 (20 units/ml) and irradiated mononuclear cells (MCs) (10^5 cells/well in 100 ml of RPMI with 10% FCS). Purified goat anti-mouse F(ab')₂ fragments (Kirkegard and Perry Laboratories, Gaithersburg, MD) are used as a crosslinking agent for the experimental conditions where monoclonal antibodies to CD3 are used. Wells are pretreated with 1.25 μ g/ml of goat anti-mouse antibody for 45 minutes at 37°C and washed three times prior to the addition of monoclonal antibodies to CD3 and CD28. Controls included T cells alone, T cells plus irradiated MCs, and T cells plus mitogenic stimuli without IL-2 or irradiated MC. After 7 days in culture at 37°C, cell proliferation are assessed using either radio-active assays or commercially available non-radioactive, ELISA based assays (e.g. Promega). Cells are co-cultured for 5-7 days to induce proliferation of the T cells (the stimulator cells are also irradiated and thus non-proliferative). Stimulator cells alone serve as controls.

An additional approach to testing T cell function uses flow cytometry based staining for intracellular expression of the cytokines IL-2 γ IFN and TNF α using antibodies specific to the human forms of these factors (Becton Dickinson). These cytokines are produced in the T progeny in the antigen specific *in vitro* proliferation assays. This allows low level detection of human cells among a high proportion of mouse cells, selectively highlighting the human progeny and excluding the mouse cells. Further, semiquantitative RT-PCR of mRNA for these factors can also be used.

In one particular example, for instance, cells removed from co-culture after 14 days showed pronounced proliferation when placed in liquid culture with complete medium and IL-2 (10 IU/mL) and phytohemagglutinin (PHA; 2 μ g/mL). After a further 7 days in culture there was a 45-fold increase in cell number: >90% were CD3⁺CD4⁺ TCR $\alpha\beta$ ⁺; 3% CD3⁺CD8⁺ TCR $\alpha\beta$ ⁺ and 3% CD3⁺CD8⁺CD4⁺ TCR $\alpha\beta$ ⁺. No cells expressing TCR $\gamma\delta$ were detected.

TABLE 2. - USE OF THYMIC STROMA/HUMAN FETAL CD34+CELL CO-CULTURE SYSTEM

	Standard Protocol n=3 Human UCB CD34+ Human fetal thymic stroma		Fetal Human Thymic stroma	6 Week Murine Thymic stroma	Neonatal swine Thymic stroma	Fetal Human Thymic stroma	6 Week Murine Thymic stroma	Neonatal Swine Thymic stroma
	d14	d28	n=3 Cell foam Thy CD34+	n=3 Cell foam Thy CD34+	n=3 Cell foam Thy CD34+	n=3 Monolayer Thy CD34+	n=3 Monolayer Thy CD34+	n=3 Monolayer Thy CD34+
CD4	2.8	3.8	81.54	85.8	67.8	88.2	81.1	69.2
CD3	1.5	1.1	12.75	87.1	80.7	13.1	90.1	79.2
CD4/CD3	4.2	1.4	9.83	75.2	64.65	10.6	68.1	66.3
CD8/CD3	2.1	2.8	0.13	74.5	28.8	0.46	71.9	28.3
CD4/CD8	1.8	1	0.37	79.5	25.4	0.89	89.1	22.9
CD2	4.1	12.5	ND	23.2	50.7	ND	20.4	48.1
CD14	33.4	16.1	59.6	0.17	6.8	63.1	0.81	4.6
CD33	48.2	20.2	90.61	0.33	ND	84.1	0.59	ND
CD2/CD14	1.6	7.3	ND	0.85	ND	ND	0.26	ND
CD2/CD33	4.8	12.8	ND	3.76	ND	ND	4.12	ND
CD33/CD14		8.4	60.73	0.13	ND	56.13	0.11	ND
Viable Cell Count								
t ₀ =5,000	195,000	210,000	98,000	1,800,000	220,000	15,000	79,000	51,000

Example 4: T Cell Lymphopoiesis Assay

AC133⁺ progenitor cells were added to the murine thymic stromal cultures at cell densities of either 1×10^5 , 1×10^4 , or 1×10^3 cells per well and cultured for an additional two weeks at 37°C in a 5% CO₂ humidified atmosphere. Medium in the co-cultures was changed every 4 days and was not supplemented with exogenous cytokines. Cells generated from the precursors were harvested 7 and 14 days after establishment of the co-cultures.

The selected AC133⁺ cells represented a highly purified progenitor cell population. Immunophenotypic analysis showed that >98% were CD34⁺; none co-expressed surface CD3, CD4 or CD8. A small number of contaminating CD2⁺ cells were detectable by flow cytometry within the AC133⁺ selected population: this comprised only $0.57\% \pm 0.29\%$ (mean \pm SEM; n=6) of the cells obtained from the selection process.

Example 5: Determination of Optimum Matrix Size and Input Cell Number

Having tested matrices of differing dimensions we have determined that the optimal sized matrix for use in this system measures 10mm diameter x 1 mm in depth. Similarly, input cell density appears critical for optimum T-cell generation: no lymphocytes were generated using an input cell density of less than 1×10^4 cells per well. However, using 10 x 1 mm matrices and input cell densities of 1×10^4 or 1×10^5 , we were able to generate large numbers of human cells, $71.21\% \pm 9.87\%$ (mean SEM; n=7) of which were CD3⁺, after 14 days in co-culture.

Example 6: Intra- and Inter-Sample Variability in Numbers of T-Cells Generated

In order to determine the variation of T-cell output within a given source of progenitors, multiple co-cultures were established using a single source of AC133⁺ cells at fixed cell densities (1×10^4 cells per well) on separate 10 x 1 mm matrices of murine thymic stroma. The intrasample variation of T lymphocytes generated was analyzed by cell count using trypan blue exclusion, and by immunophenotypic analysis. Human cells were distinguished by surface expression of CD45. After 7 days in co-culture, the number of mature T-cells detected was extremely low: CD3⁺ cells represented $2.02\% \pm 0.87\%$ (mean \pm standard error) of the CD45⁺-gated population, CD3⁺CD4⁺ T-cells accounted for $1.0\% \pm 0.52\%$ of the gated population and CD3⁺CD8⁺ $0.58\% \pm 0.1\%$ of the same gated population. However, after 14 days, the numbers of T-cells were significantly higher: the proportion of

CD3⁺ cells rose to 62.16% ± 4.53%; and the percentages of CD3⁺CD4⁺ and CD3⁺CD8⁺ were 42.7% ± 2.87% and 22.39% ± 1.29% respectively. These data are represented graphically in figure 2.

The intersample variation was calculated by comparing the number of T-cells generated from separate sources of CD34⁺ progenitors. In each case a fixed number of cells (1 x 10⁴ cells per well) had been introduced into co-culture. Immunophenotypic analysis of cells generated after 7 days in co-culture showed that, of the CD45 gated population, 1.57% ± 0.97% of cells expressed CD3; 2.27% ± 2.70% co-expressed CD3 and CD4; and 0.46% ± 0.23% expressed both CD3 and CD8. After 14 days, the immunophenotype of the cells harvested revealed that 71.21% ± 9.87% were CD3⁺; 37.44% ± 8.44% were CD3⁺CD4⁺, and 38.06% ± 19.13% were CD3⁺CD8⁺ as shown in figure 3.

These data demonstrate a high level of reproducibility within the system that suggests its potential in comparative analyses of input populations.

Example 7: Analysis for TCR Excision Circles (TREC)

The TCR *Vδ* locus lies between the TCR *Vα* and TCR *Jα* segments. In order to complete TCRα *VD-J* rearrangement, the TCR *Vδ* segment is excised: the 3' and 5' ends of the gene unite to form an extra-chromosomal circle of DNA termed a TCR excision circle (TREC) (Berenson RJ, *et al.*, *J Clin Invest*, 1988, 81: 951-5; Broxmeyer HE, *et al.*, *Proc Natl Acad Sci USA*, 1989, 86:3828-32). TRECs do not duplicate when the T-cell divides (Blom B., *et al.*, *J Immunol*, 1997, 158:3571-7). As a consequence, TREC levels are highest in recent thymic emigrants but are sequentially diluted amongst the emigrants' progeny. TCRδ TRECs are detectable by PCR - an assay that has been shown to be a reliable tool for monitoring *de novo* T-cell generation (Tjormford GE, *et al.*, *J Exp Med*, 1993, 177:1531-9). Absolute numbers of TREC positive cells will vary according to the total number of cells analyzed. We determined that the significance of TREC positivity would be most fairly interpreted by calculating the ratio of the number of TREC copies detected to the number of β-actin copies detected. We compared the level of TREC detected in T-cells harvested from the co-cultures after 14 days to TREC levels in peripheral blood mononuclear cells, B cells, AC133⁺ cells from the input population, and human fetal thymocytes. The highest TREC:βactin ratio was found in T-cells generated from the co-cultures after 14 days (0.54), followed by thymocytes from 16-22 week human fetuses (0.017). The TREC:βactin ratios from fetal and adult PBMCs

and from AC133⁺ bone marrow mononuclear cells was significantly lower. These data are summarized in Table 3, below. No TREC was detected in any of the samples of B-cells tested (n=6).

Table 3.

Source	n	TREC/Bactin ratio (mean)
Murine Thymocytes	6	0
Bone Marrow AC133+ progenitors	3	0.000014
Adult Peripheral blood MNCs	3	0.00141
Fetal Peripheral blood MNCs	1	0.0024
Fetal Thymocytes	2	0.017
T-Cells generated <i>in vitro</i>	2	0.54

These data conclusively demonstrate that rearrangement of TCR occurs during the course of the culture period. The abundance of TREC positive cells compares favorably with that seen from fresh fetal thymus and supports the physiologic equivalence of the *in vitro* system in this aspect of T-cell differentiation.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references disclosed herein are incorporated by reference in their entirety.

We claim:

Claims

1. A method for *in vitro* production of lymphoid tissue-specific cells, comprising:

introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix, wherein the amount of the lymphoreticular stromal cells is sufficient to support the growth and differentiation of the hematopoietic progenitor cells, and

co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells.

2. The method of claim 1, wherein the co-culturing occurs under conditions sufficient to produce at least a 10-fold increase in the number of lymphoid tissue origin cells.

3. The method of claim 1, wherein the co-culturing occurs under conditions sufficient to produce at least a 20-fold increase in the number of lymphoid tissue origin cells.

4. The method of claim 1, wherein the co-culturing occurs under conditions sufficient to produce at least a 50-fold increase in the number of lymphoid tissue origin cells.

5. The method of claim 1, wherein the co-culturing occurs under conditions sufficient to produce at least a 100-fold increase in the number of lymphoid tissue origin cells.

6. The method of claim 1, wherein the co-culturing occurs under conditions sufficient to produce at least a 200-fold increase in the number of lymphoid tissue origin cells.

7. The method of claim 1, wherein the co-culturing occurs under conditions sufficient to produce at least a 400-fold increase in the number of lymphoid tissue origin cells.

8. The method of claim 1, wherein the hematopoietic progenitor cells are selected from the group consisting of pluripotent stem cells, multipotent progenitor cells and progenitor cells committed to specific hematopoietic lineages.

9. The method of claim 8, wherein the progenitor cells committed to specific hematopoietic lineages are committed to a lineage selected from the group consisting of T cell lineage, B cell lineage, dendritic cell lineage, Langerhans cell lineage and lymphoid tissue-specific macrophage cell lineage.

10. The method of claim 1, wherein the hematopoietic progenitor cells are derived from tissue selected from the group consisting of bone marrow, peripheral blood, umbilical cord

blood, placental blood, lymphoid soft tissue, fetal liver, embryonic cells and aortal-gonadal-mesonephros derived cells.

11. The method of claim 10, wherein the lymphoid soft tissue is selected from the group consisting of thymus, spleen, liver, lymph node, skin, tonsil and Peyer's patches.

12. The method of claim 9, wherein the lymphoreticular stromal cells are thymic stromal cells and the progenitor cells committed to specific hematopoietic lineages are committed to a T cell lineage.

13. The method of claim 1, wherein the hematopoietic progenitor cells are genetically altered hematopoietic progenitor cells.

14. The method of claim 1, wherein the lymphoreticular stromal cells are derived from at least one lymphoid soft tissue selected from the group consisting of thymus, spleen, liver, lymph node, skin, tonsil and Peyer's patches, and combinations thereof.

15. The method of claim 1, wherein the lymphoreticular stromal cells are genetically altered lymphoreticular stromal cells.

16. The method of claim 1, wherein the lymphoreticular stromal cells are seeded prior to inoculating the hematopoietic progenitor cells.

17. The method of claim 1, wherein the lymphoreticular stromal cells are seeded at the same time as the hematopoietic progenitor cells.

18. The method of claim 1, wherein the hematopoietic progenitor cells are of human origin and the lymphoreticular stromal cells are of human origin.

19. The method of claim 1, wherein the hematopoietic progenitor cells are of human origin and the lymphoreticular stromal cells are of nonhuman origin.

20. The method of claim 19, wherein the nonhuman origin lymphoreticular stromal cells are of murine origin.

21. The method of claims 1-20, wherein the porous solid matrix is an open cell porous matrix having a percent open space of at least 75%.

22. The method of claim 21, wherein the porous solid matrix has pores defined by interconnecting ligaments having a diameter at midpoint, on average, of less than 150 μ m.

23. The method of claim 22, wherein the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material.

24. The method of claim 23, wherein the metal is selected from the group consisting of tantalum, titanium, platinum, niobium, hafnium, tungsten, and combinations thereof, wherein

said metal is coated with a biological agent selected from the group consisting of collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitrogen, antibodies and fragments thereof, and combinations thereof.

25. The method of claim 24, wherein the porous, solid matrix having seeded
5 hematopoietic progenitor cells and their progeny, and lymphoreticular stromal cells, is impregnated with a gelatinous agent that occupies pores of the matrix.

26. The method of claim 24, wherein the metal is tantalum.

27. The method of claim 1, wherein the hematopoietic progenitor cells and the
10 lymphoreticular stromal cells are cultured in an environment that is free of stromal cell conditioned medium and exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion, and differentiation, other than serum.

28. The method of claim 27, wherein the hematopoietic growth factors that promote
15 hematopoietic cell maintenance, expansion, and differentiation, are agents selected from the group consisting of interleukin 3, interleukin 6, interleukin 11, SCF, FLT/FLK ligand growth factors.

29. The method of claim 1, wherein the hematopoietic progenitor cells and the
20 lymphoreticular stromal cells are cultured with an exogenously added agent selected from the group consisting of stromal cell conditioned medium, and a hematopoietic growth factor that promotes hematopoietic cell maintenance, expansion, differentiation, and influences cell localization.

30. The method of claim 29, wherein the hematopoietic growth factor that promotes
25 hematopoietic cell maintenance, expansion, differentiation, and influences cell localization, is an agent selected from the group consisting of interleukin 3, interleukin 6, interleukin 7, interleukin 11, interleukin 12, stem cell factor, FLK-2 ligand, FLT-2 ligand, Epo, Tpo, GMCSF, GCSF, Oncostatin M, and MCSF.

31. The method of claim 1, further comprising:

after the co-culturing step, harvesting the lymphoid tissue origin cells.

32. A method for *in vivo* maintenance, expansion and/or differentiation of hematopoietic
progenitor cells, comprising:

30 implanting into a subject a porous, solid matrix having seeded hematopoietic progenitor cells and lymphoreticular stromal cells,

wherein the porous, solid matrix is an open cell porous matrix having a percent open space of at least 75%.

33. The method of claim 32, further comprising the porous, solid matrix having seeded hematopoietic progenitor cells and lymphoreticular stromal cells by the steps of:

5 introducing *in vitro* an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into the porous, solid matrix;

co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells in an environment that is free of stromal cell conditioned medium and exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion and/or differentiation, other than serum.

10 34. The method of claim 33, wherein the co-culturing occurs under conditions sufficient to produce an increase in the number of lymphoid tissue origin cells of at least between 10-fold and 400-fold.

35. The method of claim 32, wherein the hematopoietic progenitor cells are selected from the group consisting of pluripotent stem cells, multipotent progenitor cells and progenitor cells committed to specific hematopoietic lineages.

15 36. The method of claim 35, wherein the progenitor cells committed to specific hematopoietic lineages are committed to a lineage selected from the group consisting of T cell lineage, B cell lineage, dendritic cell lineage, Langerhans cell lineage and lymphoid tissue-specific macrophage cell lineage.

20 37. The method of claim 36, wherein the lymphoreticular stromal cells are thymic stromal cells and the progenitor cells committed to specific hematopoietic lineages are committed to a T cell lineage.

38. The method of claim 32, wherein the porous solid matrix has pores defined by interconnecting ligaments having a diameter at midpoint, on average, of less than 150 μ m.

25 39. The method of claim 38, wherein the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material.

40. The method of claim 39, wherein the metal is selected from the group consisting of tantalum, titanium, platinum, niobium, hafnium, tungsten, and combinations thereof, wherein said metal is coated with a biological agent selected from the group consisting of collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors,

glycosaminoglycans, vitrogen, antibodies and fragments thereof, and combinations thereof.

41. The method of claim 40, wherein the metal is tantalum.

42. The method of claims 32-41, wherein the porous, solid matrix having seeded
5 hematopoietic progenitor cells and lymphoreticular stromal cells is impregnated with a gelatinous agent that occupies pores of the matrix.

43. A method for inducing T cell anergy comprising:

introducing an amount of hematopoietic progenitor cells, an amount of
antigen presenting cells and an amount of lymphoreticular stromal cells into a porous, solid
10 matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix, and

co-culturing the hematopoietic progenitor cells, the antigen presenting
cells and the lymphoreticular stromal cells in the presence of at least one antigen under
conditions sufficient to induce the formation of T cells and/or T cell progenitors and to inhibit
15 activation of the formed cells.

44. The method of claim 43, wherein the hematopoietic progenitor cells are selected from
the group consisting of pluripotent stem cells, multipotent progenitor cells, and progenitor
cells committed to specific hematopoietic lineages.

45. The method of claim 44, wherein the lymphoreticular stromal cells are thymic stromal
20 cells and the progenitor cells committed to specific hematopoietic lineages are committed to a T cell lineage.

46. A method for inducing T cell reactivity comprising:

introducing an amount of hematopoietic progenitor cells, an amount of antigen
presenting cells and an amount of lymphoreticular stromal cells into a porous, solid matrix
25 having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix, and

co-culturing the hematopoietic progenitor cells, the antigen presenting cells
and the lymphoreticular stromal cells in the presence of at least one antigen under conditions
sufficient to induce from the hematopoietic progenitor cells the formation of T cells and/or T
30 cell progenitors having specificity for the at least one antigen.

47. The method of claim 46, wherein the hematopoietic progenitor cells are selected from the group consisting of pluripotent stem cells, multipotent progenitor cells, and progenitor cells committed to specific hematopoietic lineages.

48. The method of claim 47, wherein the lymphoreticular stromal cells are thymic stromal cells and the progenitor cells committed to specific hematopoietic lineages are committed to a T cell lineage.

49. The method of claim 46, wherein the antigen presenting cells are cells selected from the group consisting of dendritic cells, monocytes/macrophages, Langerhans cells, Kupfer cells, microglia, alveolar macrophages and B cells.

50. The method of claim 46, wherein the antigen presenting cells are derived from hematopoietic progenitor cells *in vitro*.

51. The method of claim 46, further comprising administering a co-stimulatory molecule to the co-culture.

52. The method of claim 51, wherein the co-stimulatory molecule is selected from the group consisting of lymphocyte function associated antigen 3 (LFA-3), CD2, CD40, CD80/B7-1, CD86/B7-2, OX-2, CD70, and CD82.

53. A composition comprising:

a porous, solid matrix having a percent open space of at least 75% and pores of a pore size sufficient to permit cells to grow throughout the matrix,

an amount of lymphoreticular stromal cells attached to the solid matrix, wherein the amount is sufficient to support the growth and differentiation of hematopoietic progenitor cells, and

an amount of hematopoietic progenitor cells attached to the matrix.

54. The composition of claim 53, wherein the hematopoietic progenitor cells are attached to the lymphoreticular stromal cells.

55. The composition of claims 53 or 54, wherein the porous solid matrix has pores defined by interconnecting ligaments having a diameter at midpoint, on average, of less than 150µm.

56. The composition of claim 55, wherein the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material.

57. The composition of claim 56, wherein the metal is selected from the group consisting of tantalum, titanium, platinum, niobium, hafnium, tungsten, and combinations thereof, wherein said metal is coated with a biological agent selected from the group consisting of

collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitrogen, antibodies and fragments thereof, and combinations thereof.

58. The composition of claim 56, wherein the metal is tantalum.

5 59. The composition of claim 53, wherein the porous solid matrix having seeded hematopoietic progenitor cells and lymphoreticular stromal cells is impregnated with a gelatinous agent that occupies pores of the matrix.

60. The composition of claim 53, wherein the hematopoietic progenitor cells are selected from the group consisting of pluripotent stem cells, multipotent progenitor cells and
10 progenitor cells committed to specific hematopoietic lineages.

61. The composition of claim 60, wherein the progenitor cells committed to specific hematopoietic lineages are committed to a lineage selected from the group consisting of T cell lineage, B cell lineage, dendritic cell lineage, Langerhans cell lineage and lymphoid tissue-specific macrophage cell lineage.

15 62. The composition of claim 60, wherein the lymphoreticular stromal cells are thymic stromal cells and the progenitor cells committed to specific hematopoietic lineages are committed to a T cell lineage.

63. A method for identifying an agent suspected of affecting hematopoietic cell development, comprising:

20 introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix,

25 co-culturing in a test co-culture the hematopoietic progenitor cells and the lymphoreticular stromal cells in the presence of at least one candidate agent suspected of affecting hematopoietic cell development, and

determining whether the at least one candidate agent affects hematopoietic cell development in the test co-culture by comparing the test co-culture hematopoietic cell development to a control co-culture whereby hematopoietic progenitor cells and
30 lymphoreticular stromal cells are co-cultured in the absence of the at least one candidate agent,

wherein the porous, solid matrix is an open cell porous matrix having a percent open space of at least 75%.

64. The method of claim 63, wherein hematopoietic progenitor cell development comprises hematopoietic progenitor cell maintenance.

5 65. The method of claim 63, wherein hematopoietic progenitor cell development comprises hematopoietic progenitor cell expansion.

66. The method of claim 63, wherein hematopoietic progenitor cell development comprises hematopoietic progenitor cell differentiation toward a specific cell lineage.

67. The method of claim 63, wherein hematopoietic progenitor cell development
10 comprises hematopoietic progenitor cell-death.

68. The method of claim 63, wherein the lymphoreticular stromal cells are thymic stromal cells.

69. The method of claim 63, wherein the porous solid matrix has pores defined by interconnecting ligaments having a diameter at midpoint, on average, of less than 150 μ m.

15 70. The method of claim 63, wherein the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material.

71. The method of claim 69, wherein the metal is selected from the group consisting of tantalum, titanium, platinum, niobium, hafnium, tungsten, and combinations thereof, wherein said metal is coated with a biological agent selected from the group consisting of collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors,
20 glycosaminoglycans, vitrogen, antibodies and fragments thereof, and combinations thereof.

72. The method of claim 70, wherein the metal is tantalum.

73. A method for isolating from a cell culture an agent suspected of affecting
25 hematopoietic cell development, comprising:

introducing an amount of hematopoietic progenitor cells and an amount of lymphoreticular stromal cells into a porous, solid matrix having interconnected pores of a pore size sufficient to permit the hematopoietic progenitor cells and the lymphoreticular stromal cells to grow throughout the matrix,

30 co-culturing the hematopoietic progenitor cells and the lymphoreticular stromal cells,

obtaining a test-supernatant from the co-culture,

comparing the test-supernatant to a control-supernatant, and
obtaining a subfraction of the test-supernatant that contains an agent suspected
of affecting hematopoietic cell development that is absent from the control-supernatant,

wherein the porous, solid matrix is an open cell porous matrix having a percent open
space of at least 75%.

74. The method of claim 73, wherein the agent is present in the control-supernatant and
absent from the test-supernatant.

75. The method of claim 73, wherein the agent in the test-supernatant is different from the
agent in the control-supernatant.

76. The method of claim 73, wherein the agent is further isolated by preparing a further
subfraction of the test-supernatant.

77. The method of claim 73, wherein hematopoietic progenitor cell development
comprises hematopoietic progenitor cell maintenance.

78. The method of claim 73, wherein hematopoietic progenitor cell development
comprises hematopoietic progenitor cell expansion.

79. The method of claim 73, wherein hematopoietic progenitor cell development
comprises hematopoietic progenitor cell differentiation toward a specific cell lineage.

80. The method of claim 73, wherein hematopoietic progenitor cell development
comprises hematopoietic progenitor cell-death.

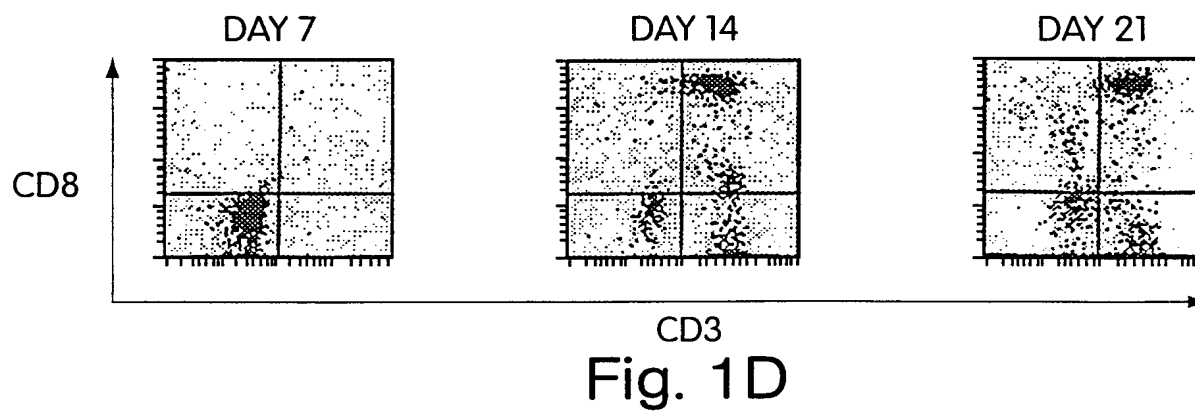
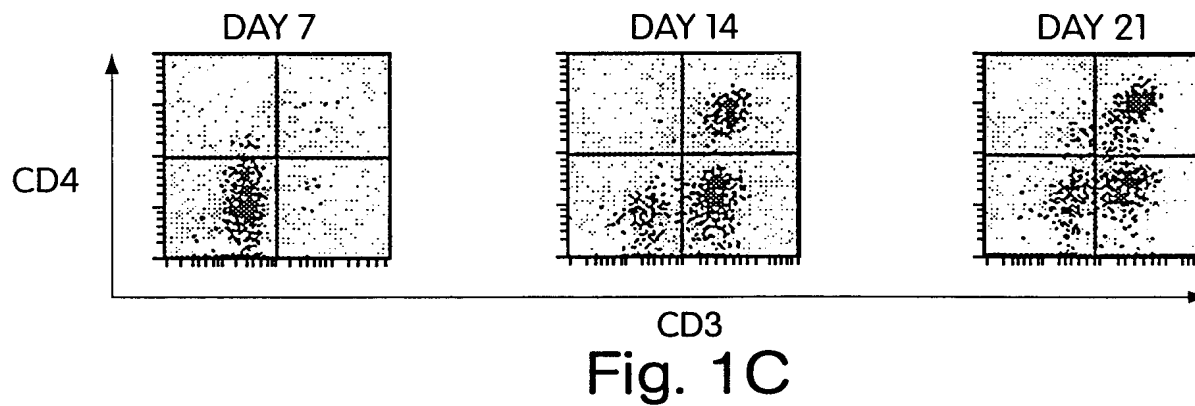
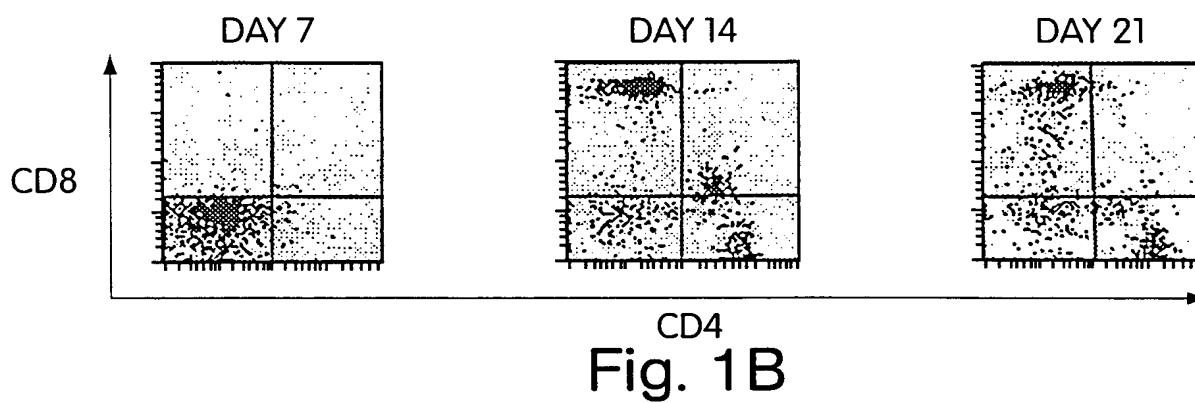
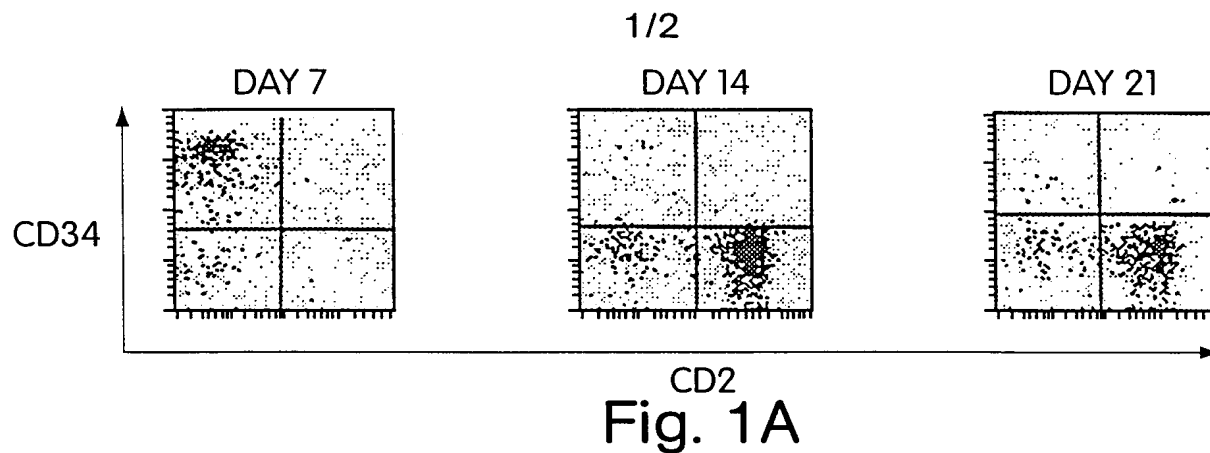
81. The method of claim 73, wherein the lymphoreticular stromal cells are thymic stromal
cells.

82. The method of claim 73, wherein the porous solid matrix has pores defined by
interconnecting ligaments having a diameter at midpoint, on average, of less than 150 μ m.

83. The method of claim 73, wherein the porous solid matrix is a metal-coated reticulated
open cell foam of carbon containing material.

84. The method of claim 83, wherein the metal is selected from the group consisting of
tantalum, titanium, platinum, niobium, hafnium, tungsten, and combinations thereof, wherein
said metal is coated with a biological agent selected from the group consisting of collagens,
fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors,
glycosaminoglycans, vitrogen, antibodies and fragments thereof, and combinations thereof.

85. The method of claim 83, wherein the metal is tantalum.



2/2

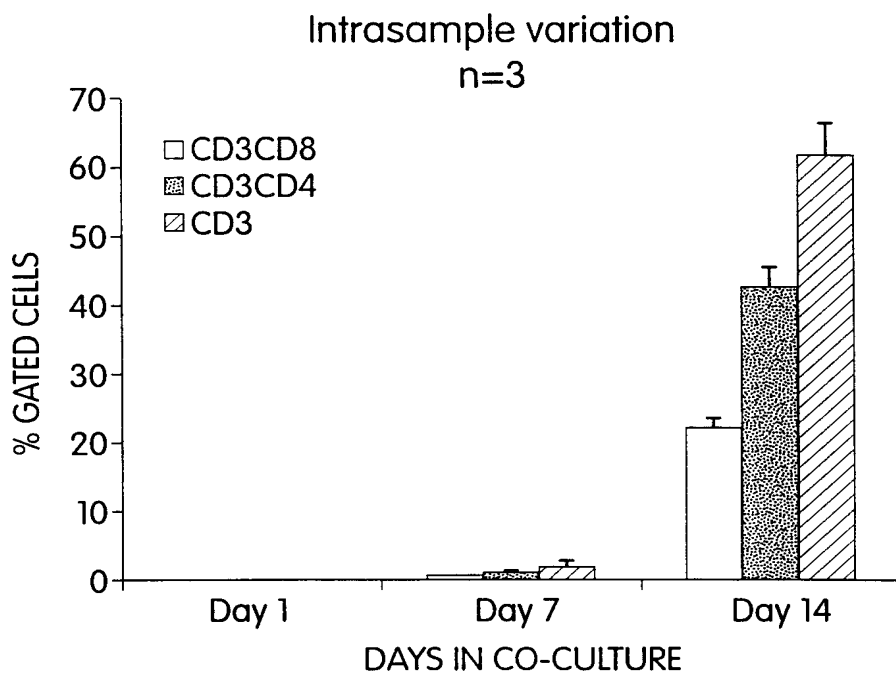


Fig. 2

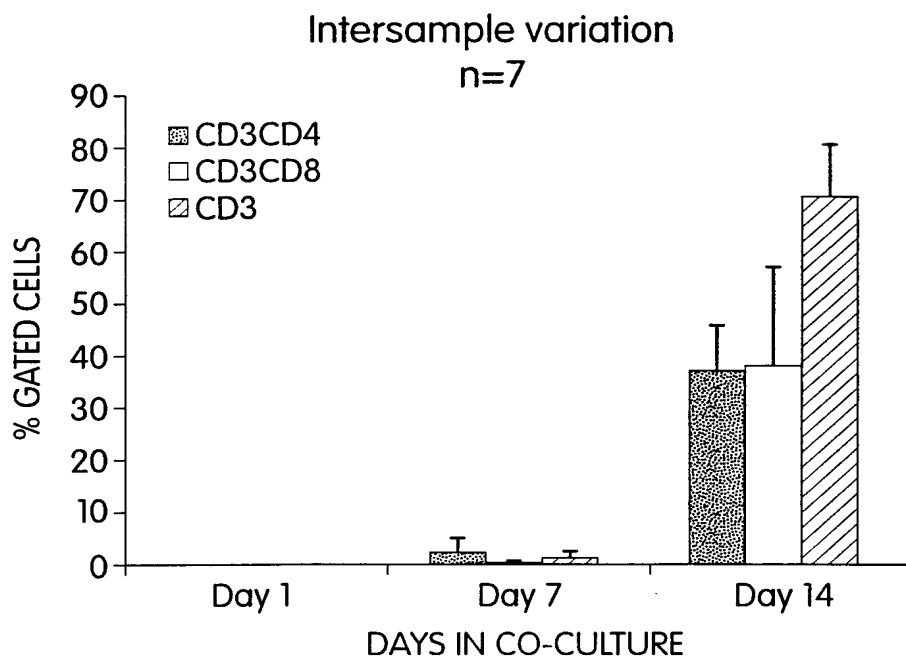


Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/26795

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N5/06 C12N5/08 A61L27/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NAUGHTON G ET AL: "Three-dimensional bone marrow cell and tissue culture system" BIOTECHNOLOGY ADVANCES, GB, ELSEVIER PUBLISHING, BARKING, vol. 15, no. 2, 1 January 1997 (1997-01-01), page 401 XP004073886 ISSN: 0734-9750 the whole document	1-85
X	EP 0 358 506 A (MARROW TECH INC) 14 March 1990 (1990-03-14) abstract page 3, line 5 -page 3, line 40; claims 1-133 ----- -/-	1-85



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

Date of the actual completion of the international search

15 May 2000

Date of mailing of the international search report

25 May 2000 (25.05.00)

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Meyer, W

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/26795

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 443 950 A (NAUGHTON GAIL K ET AL) 22 August 1995 (1995-08-22) abstract ---	1-85
X	EP 0 241 578 A (MARROW GROUP INT) 21 October 1987 (1987-10-21) abstract; claims 1-8 ---	1-85
X	US 5 580 781 A (NAUGHTON GAIL K ET AL) 3 December 1996 (1996-12-03) abstract; page 3, line 34 -page 4, line 21; claims 1-50 ---	1-85
X	WO 90 15877 A (UNIV MICHIGAN) 27 December 1990 (1990-12-27) abstract; claims 1-19 ---	1-85
X	NAUGHTON B A ET AL: "A THREE-DIMENSIONAL CULTURE SYSTEM FOR THE GROWTH OF HEMA- TOPOIETIC CELLS" PROGRESS IN CLINICAL AND BIOLOGICAL RESEARCH, US, NEW YORK, NY, vol. 333, 1990, pages 435-445, XP000881141 ISSN: 0361-7742 the whole document ---	1-31
X	ROSENZWEIG M ET AL: "Enhanced maintenance and retroviral transduction of primitive hematopoietic progenitor cells using a novel three-dimensional culture system" GENE THERAPY, GB, MACMILLAN PRESS LTD., BASINGSTOKE, vol. 4, no. 9, 1 September 1997 (1997-09-01), pages 928-936, XP002091698 ISSN: 0969-7128 abstract ---	1-85
A	BAGLEY J ET AL: "LONG-TERM THREE DIMENSIONAL HEMATOPOIETIC STEM CELL CULTURE" AMERICAN CHEMICAL SOCIETY. ABSTRACTS OF PAPER. AT THE NATIONAL MEETING, US, AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC, vol. 216, no. 1/03, 1998, page B10T129 XP000881132 ISSN: 0065-7727 abstract --- -/--	1-85

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/26795

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>BAGLEY J ET AL: "EXTENDED CULTURE OF MULTIPOTENT HEMATOPOIETIC PROGENITORS WITHOUT CYTOKINE AUGMENTATION IN A NOVEL THREE-DIMENSIONAL DEVICE"</p> <p>EXPERIMENTAL HEMATOLOGY, US, NEW YORK, NY, vol. 27, no. 3, March 1999 (1999-03), pages 496-504, XP000881139</p> <p>ISSN: 0301-472X</p> <p>the whole document -----</p>	1-85

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/26795

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 32-42 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/26795

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0358506 A	14-03-1990	US 4963489 A	16-10-1990
		US 5032508 A	16-07-1991
		AU 644578 B	16-12-1993
		AU 4211489 A	02-04-1990
		BR 8907642 A	20-08-1991
		CA 1335657 A	23-05-1995
		DK 40591 A	07-05-1991
		HU 56393 A	28-08-1991
		IL 91536 A	31-10-1996
		JP 4501657 T	26-03-1992
		KR 156571 B	15-10-1998
		KR 156684 B	15-10-1998
		KR 156685 B	15-10-1998
		NO 910787 A	22-04-1991
		NZ 230572 A	23-12-1993
		PT 91676 A	30-03-1990
		WO 9002796 A	22-03-1990
		US 5443950 A	22-08-1995
		US 5962325 A	05-10-1999
		US 5460939 A	24-10-1995
		US 5510254 A	23-04-1996
		US 6022743 A	08-02-2000
		US 5580781 A	03-12-1996
		US 5516680 A	14-05-1996
		US 5512475 A	30-04-1996
		US 5541107 A	30-07-1996
		US 5516681 A	14-05-1996
		US 5578485 A	26-11-1996
		US 5785964 A	28-07-1998
		US 5518915 A	21-05-1996
		US 5624840 A	29-04-1997
		US 5902741 A	11-05-1999
		US 5863531 A	26-01-1999
		US 5266480 A	30-11-1993
		US 5160490 A	03-11-1992
		US 5849588 A	15-12-1998
		US 5858721 A	12-01-1999
		ZA 8906886 A	27-06-1990
US 5443950 A	22-08-1995	US 5266480 A	30-11-1993
		US 5032508 A	16-07-1991
		US 4963489 A	16-10-1990
		US 4721096 A	26-01-1988
		US 5962325 A	05-10-1999
		US 5460939 A	24-10-1995
		US 5510254 A	23-04-1996
		US 6022743 A	08-02-2000
		US 5580781 A	03-12-1996
		US 5516680 A	14-05-1996
		US 5512475 A	30-04-1996
		US 5541107 A	30-07-1996
		US 5516681 A	14-05-1996
		US 5578485 A	26-11-1996
		US 5785964 A	28-07-1998
		US 5518915 A	21-05-1996
		US 5624840 A	29-04-1997
		US 5902741 A	11-05-1999
		US 5863531 A	26-01-1999

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/26795

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5443950 A		US 5849588 A	15-12-1998
		US 5858721 A	12-01-1999
		AU 644578 B	16-12-1993
		AU 4211489 A	02-04-1990
		BR 8907642 A	20-08-1991
		CA 1335657 A	23-05-1995
		DK 40591 A	07-05-1991
		EP 0358506 A	14-03-1990
		HU 56393 A	28-08-1991
		IL 91536 A	31-10-1996
		JP 4501657 T	26-03-1992
		KR 156571 B	15-10-1998
		KR 156684 B	15-10-1998
		KR 156685 B	15-10-1998
		NO 910787 A	22-04-1991
		NZ 230572 A	23-12-1993
		PT 91676 A	30-03-1990
		WO 9002796 A	22-03-1990
		US 5160490 A	03-11-1992
		ZA 8906886 A	27-06-1990
		AT 127692 T	15-09-1995
		AU 6815990 A	14-03-1991
		AU 6816090 A	14-03-1991
		AU 615414 B	03-10-1991
		AU 7356887 A	09-11-1987
		BG 51337 A	15-04-1993
		BR 8707673 A	15-08-1989
		CA 1310926 A	01-12-1992
		DE 3751519 D	19-10-1995
		DK 665687 A	17-12-1987
EP 0241578 A	21-10-1987	AT 127692 T	15-09-1995
		AU 595813 B	12-04-1990
		AU 5985086 A	22-10-1987
		AU 6815990 A	14-03-1991
		AU 6816090 A	14-03-1991
		AU 615414 B	03-10-1991
		AU 7356887 A	09-11-1987
		BG 51337 A	15-04-1993
		BR 8707673 A	15-08-1989
		CA 1282725 A	09-04-1991
		CA 1310926 A	01-12-1992
		DE 3751519 D	19-10-1995
		DK 665687 A	17-12-1987
		EP 0309456 A	05-04-1989
		FI 884783 A	17-10-1988
		HK 1007684 A	23-04-1999
		HU 48112 A	29-05-1989
		JP 62249926 A	30-10-1987
		JP 10114664 A	06-05-1998
		JP 1503195 T	02-11-1989
		JP 2857392 B	17-02-1999
		KR 142885 B	15-07-1998
		NO 179181 B	13-05-1996
		NZ 218282 A	28-05-1990
		NZ 220038 A	28-08-1990
		RO 106655 A	30-06-1993
		US 4721096 A	26-01-1988

INTERNATIONAL SEARCH REPORT

Information on patent family members

I. International Application No

PCT/US 99/26795

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0241578 A		WO 8706120 A	22-10-1987
		US 5443950 A	22-08-1995
		US 5962325 A	05-10-1999
		US 5460939 A	24-10-1995
		US 5510254 A	23-04-1996
		US 6022743 A	08-02-2000
		US 5580781 A	03-12-1996
		US 5516680 A	14-05-1996
		US 5512475 A	30-04-1996
		US 5541107 A	30-07-1996
		US 5516681 A	14-05-1996
		US 5578485 A	26-11-1996
		US 5785964 A	28-07-1998
		US 5518915 A	21-05-1996
		US 5624840 A	29-04-1997
		US 5902741 A	11-05-1999
		US 5863531 A	26-01-1999
		US 5266480 A	30-11-1993
		US 5160490 A	03-11-1992
		US 5849588 A	15-12-1998
		US 5858721 A	12-01-1999
US 5580781 A	03-12-1996	US 5443950 A	22-08-1995
		US 5266480 A	30-11-1993
		US 5032508 A	16-07-1991
		US 4963489 A	16-10-1990
		US 4721096 A	26-01-1988
		US 5962325 A	05-10-1999
		US 5460939 A	24-10-1995
		US 5510254 A	23-04-1996
		US 6022743 A	08-02-2000
		US 5516680 A	14-05-1996
		US 5512475 A	30-04-1996
		US 5541107 A	30-07-1996
		US 5516681 A	14-05-1996
		US 5578485 A	26-11-1996
		US 5785964 A	28-07-1998
		US 5518915 A	21-05-1996
		US 5624840 A	29-04-1997
		US 5902741 A	11-05-1999
		US 5863531 A	26-01-1999
		US 5849588 A	15-12-1998
		US 5858721 A	12-01-1999
		AU 644578 B	16-12-1993
		AU 4211489 A	02-04-1990
		BR 8907642 A	20-08-1991
		CA 1335657 A	23-05-1995
		DK 40591 A	07-05-1991
		EP 0358506 A	14-03-1990
		HU 56393 A	28-08-1991
		IL 91536 A	31-10-1996
		JP 4501657 T	26-03-1992
		KR 156571 B	15-10-1998
		KR 156684 B	15-10-1998
		KR 156685 B	15-10-1998
		NO 910787 A	22-04-1991
		NZ 230572 A	23-12-1993
		PT 91676 A	30-03-1990

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. National Application No

PCT/US 99/26795

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5580781 A		WO 9002796 A	22-03-1990
		US 5160490 A	03-11-1992
		ZA 8906886 A	27-06-1990
		AT 127692 T	15-09-1995
		AU 6815990 A	14-03-1991
		AU 6816090 A	14-03-1991
		AU 615414 B	03-10-1991
		AU 7356887 A	09-11-1987
		BG 51337 A	15-04-1993
		BR 8707673 A	15-08-1989
		CA 1310926 A	01-12-1992
		DE 3751519 D	19-10-1995
		DK 665687 A	17-12-1987
WO 9015877 A	27-12-1990	AT 148502 T	15-02-1997
		CA 2062741 A	16-12-1990
		DE 69029856 D	13-03-1997
		DE 69029856 T	04-09-1997
		DK 477290 T	21-07-1997
		EP 0477290 A	01-04-1992
		EP 0753574 A	15-01-1997
		ES 2097149 T	01-04-1997
		JP 4506153 T	29-10-1992
		US 5437994 A	01-08-1995
		US 5459069 A	17-10-1995
		US 5763266 A	09-06-1998
		US 5635386 A	03-06-1997
		US 5605822 A	25-02-1997
		US 5670147 A	23-09-1997
		US 5670351 A	23-09-1997
		US 5646043 A	08-07-1997
		US 5888807 A	30-03-1999
		US 5399493 A	21-03-1995